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DOCTOR OF PHILOSOPHY

Chromatin Dynamics During DNA Replication

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Chromatin Dynamics During DNA Replication

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Abstract

The primary level of chromatin organisation consists of arrays of nucleosomes that are present across the genetic template. Advances in the post genomics era have made it possible to determine the positions of nucleosomes genome-wide where it has been observed that nucleosomes adopt a distinct organisation with respect to genetic and trans-binding elements. Amongst the best studied of these is the transcription start site where it has been observed that genic nucleosome locations are well maintained with respect to promoters.

DNA and chromatin replication are coupled processes whereby chromatin is disrupted ahead of the replication fork and nucleosomes are rapidly assembled on the nascent DNA template. Classically it has been observed that nascent chromatin is more susceptible to digestion, prompting the possibility of an “immature” chromatin organisation post assembly. However it has not been investigated genome-wide if nucleosomes are initially assembled in phase or must be reorganised post assembly to canonical locations.

We have developed two methods of isolating nascent DNA fragments representative of nucleosome positions from synchronised and asynchronous populations of the budding yeast *S. cerevisiae*. High throughput sequencing has revealed that chromatin is assembled and organised rapidly behind the replication fork. The most nascent chromatin isolated displays typical patterns of nucleosome organisation suggesting that reorganisation of nucleosomes on the nascent template is replication coupled. Deletion of specific histone chaperones and chromatin remodelers perturbs this pathway. However, this can be compensated for by a transcription directed reorganisation of nascent chromatin. Our analysis of nascent chromatin has allowed

us to investigate the mechanisms that act to direct chromatin organisation in addition to evaluation of models that describe nucleosome organisation genome-wide.

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I thank my parents for their continued support over my life and for allowing me to choose my own path. I also wish to thank my sister for when my parents ‘support’ becomes too much for me to bear! Also a special thanks to Bob for being there through thick and thin.

I suspect I am not an easy person to live with, with that in mind I wish to thank Monika Żwirek for tolerating my living habits, perverse sense of humour and sleeping routine but perhaps most all for being my sole source of human contact while writing this thesis!

Finally I wish to thank Cristiane and Clément Gravouil for being my first friends when I arrived in Dundee. From attempting to understand my accent to eventually claiming me as an adopted son I’m truly grateful for your feeding, housing and general toleration of me!

Declaration

I, Ross Fennessy, declare that I am the sole author of this thesis, that the research presented here is my own unless there is a clear statement otherwise. None of this research has previously been accepted for a higher degree.

Ross Fennessy

I certify that Ross Fennessy has spent the equivalent of at least nine terms in research work at the University of Dundee Wellcome Trust Centre for Gene Regulation and Expression and that he has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Tom Owen-Hughes

Abbreviations

¹² C	Carbon - 12
¹³ C	Carbon - 13
³ H	Hydrogen - 3
¹⁴ N	Nitrogen - 14
¹⁵ N	Nitrogen - 15
Abf1	ARS-Binding Factor 1
acetyl-CoA	Acetyl-coenzyme A
ACS	ARS Consensus Sequence
ARS	Autonomous Replicating Sequence
Asf1	Anti-silencing factor 1
ATM	Ataxia Teleangiectasia Mutated
ATP	Adenosine triphosphate
ATR	AminoTriazole Resistance
bp	Base pairs
BrdU	5-bromo-2'-deoxyuridine
Bre1	BREfoldin A sensitivity 1
Bur1	Sgv1, Suppressor of Gpa1 ^{Val50} mutation
Cac1	p150 subunit of CAF-1
Cac2	p60 subunit of CAF-1
Cac3	p48 subunit of CAF-1
CAF-1	Chromatin assembly factor 1
Cdc6	Cell Division Cycle 6
Cdc9	Cell Division Cycle 9
Cdc45	Cell Division Cycle 45
Cdc73	Cell Division Cycle 73
Cdk7	Cell Division Kinase 7
Cdt1	Topo-A Hypersensitive
CENP-A	Centromere Protein A
Chd1	Chromatin organization modifier, Helicase, and DNA-binding domains 1
ChIP	Chromatin Immuno-precipitation
ChIP-exo	Exo-nuclease Chromatin Immuno-precipitation
CsCl	Caesium Chloride
Cse4	Chromosome SEgregation 4
CTD	C' Terminal Domain
Ctr9	Copper Transport 9
D	Deuterium
Da	Dalton
Dbf4	DumbBell Former 4
DDK	Dbf4-dependent kinase
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
Dot1	Disrupter of telomere silencing protein 1

DSB	Double strand break
DUB	Deubiquitinase
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
FACS	Fluorescence-activated cell sorting
FACT	Facilitates chromatin transcription complex
Fun30	Function Unknown Now 30
FRET	Fluorescence Resonance Energy Transfer
Gal4	GALactose metabolism 4
Gcn5	General Control Nonderepressible 5
H1	Histone H1
H2A	Histone H2A
H2AX	Histone H2AX
H2AZ	Histone H2AZ
H2B	Histone H2B
H3	Histone H3
H3.1	Histone H3.1
H3.3	Histone H3.3
H4	Histone H4
HAT	Histone acetyl transferases
HDAC	Histone Deacetylase
hENT1	Human Equilibrative Nucleoside Transporter 1
HKMT	Histone Lysine Methyl Transferase
Hos2	Hda One Similar 2
Hst1	Homolog of SIR Two 2
Htz1	Histone Two A Z
H2B123Ub	Histone H2B ubiquitinated at lysine 123
H3K56Ac	Histone H3 acetylated at lysine 56
H3K4 me	Histone H3 methylated at lysine 4
H3K36 me	Histone H3 methylated at lysine 36
Ies5	Ino Eighty Subunit 5
INO80	INOsitol requiring 80
ISWI	Imitation-switch
Jmj	Jumonji Domain
Leo1	LEft Open reading frame
macroH2A	Histone macroH2A
MCM2-7	MiniChromosome Maintenance 2-7
NaCl	Sodium chloride
Nap1	Nucleosome assembly protein 1
NASP	Nuclear autoantigenic sperm protein
NDR	Nucleosome Depleted Region
Nhp10	Non-Histone Protein 6
Nhp10	Non-Histone Protein 10
NRI	Nitrogen regulator I
NuA3	Nucleosomal acetyltransferases of H3
NuRD	Nucleosome Remodeling Histone Deacetylase Complex

MMTV	Mouse Mammary Tumor Virus
MNase	Micrococcal Nuclease
OD ₆₆₀	Optical Density at 660nm wavelength
ORC	Origin Recognition complex
ORI	Origin of Replication
PAF Complex	RNA Polymerase II Associated Factor Complex
Paf1	RNA Polymerase II Associated Factor 1
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
Pho2	PHosphate metabolism 2
Pho4	PHosphate metabolism 4
Pho5	PHosphate metabolism 5
Pho8	PHosphate metabolism 8
Pob3	POI Binding 3
Pol δ	DNA polymerase delta
Pol ϵ	DNA polymerase epsilon
PolyA	Poly Adenine DNA Sequence
Poly (dA:dT)	Adenine/Thymidine Dinucleotide
PTM	Post Translational Modification
Pre-RC	Pre-replication Complex
Rad9	RADIation sensitive 9
Rap1	Repressor Activator Protein
RSC	Remodel the Structure of Chromatin
Rtf1	Restores TBP Function
RbAp46/48	Cac3 subunit of CAF-1
Reb1	RNA polymerase I Enhancer Binding protein
RI	Refractive Index
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
Rpd3	Reduced Potassium Dependency 3
Rtt106	Repressor of Ty1 transposition 106
Rtt109	Repressor of Ty1 transposition 109
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SAGA	Spt/Ada/Gcn5 acetyltransferase
SAM	Sequence Alignment/Map format
SAM	S-Adenosyl Methionine
SDS	Sodium dodecyl sulphate
Sec3	SECRETory 3
SET	Su(var)3-9, Enhancer-of-zeste and Trithorax
Set1	SET domain-containing 1
Set2	SET domain-containing 2
Set3	SET domain-containing 3
Sgf29	SaGa associated Factor 29
SHL	Super Helical Location

	SWI/SNF-Related, Matrix-Associated Actin-Dependent Regulator Of
SMARCAD1	Chromatin, Subfamily A, Containing DEAD/H Box 1
Snf2	Sucrose Non Fermenting 2
Spt4	SuPpressor of Ty's 4
Spt6	SuPpressor of Ty's 6
Spt16	SuPpressor of Ty's 16
SSRP1	Structure specific recognition protein 1
SWI/SNF	SWItching deficient/Sucrose non-fermenting
SWR1	Sick With Rat8 ts
Swc2	SWR1 complex subunit
TBE	Tris Borate EDTA
TF	Transcription Factor
tK	Thymidine Kinase
TFIIH	Transcription Factor II Human
TSS	Transcription Start Site
WT	Wild type
YAC	Yeast Artificial Chromosome
YPAD	Yeast extract-peptone-dextrose medium + adenine (YPAD)
Å	Angstrom
α	Alpha helix
α C	C-terminal alpha helix
α N	Histone H3 N-terminal alpha helix
γ H2A	Histone H2A phosphorylated at serine 129

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1 Introduction

1.1 Chromatin

The genetic complement of eukaryotic cells is organised as a complex of DNA/RNA and protein, termed chromatin. Initially observed by Flemming (Flemming 1878) the human genome consists of 3.2G bp of DNA in the form of 46 chromosomes which are organised in a nucleus 20-50µm in diameter. The physical constraint of housing the genome in the nucleus is in part achieved through interaction of linear DNA with the histone proteins. More significantly however this interaction mediates selective accessibility to the DNA template. The primary level of chromatin organisation is the nucleosome.

1.2 The Nucleosome Core Particle

Many properties of the nucleosome were discovered some 40 years ago when the nucleosome was initially characterised (Kornberg 1974, Kornberg and Thomas 1974). Pioneering biochemical and x-ray diffraction studies showed that chromatin consisted of near equimolar ratios of the four core histone proteins H2A, H2B, H3 and H4 of which two copies of each were present at ~ 205 bp intervals with a fifth histone, H1 present at much lower levels (Kornberg 1974, Noll 1974). This characterisation coincided with electron microscopy studies that described primary level chromatin as arrays of spheres roughly 70Å in diameter (Olins and Olins 1974). Further digestion studies demonstrated that more stringent digestion with endo-exonucleases yielded a fundamental DNA repeat of ~146 bp which forms the nucleosome core particle (Axel 1975, Lohr, Kovacic et al. 1977).

Almost two decades prior to its complete structural elucidation, much was known about how the histone proteins were organised within the context of nucleosomal DNA. High salt washing of chromatin showed that the core histones H3 and H4 formed a tetramer and associate in the core of the nucleosome (Kornberg and Thomas 1974) which undergo conformational changes when assembled *in vitro* (D'Anna and Isenberg 1974). Further cross linking studies demonstrated that H2A and H2B form dimers outside of the (H3-H4)₂ tetramer (Thomas and Kornberg 1975). Use of endonucleases demonstrated that histone DNA contacts were non continuous at ~10 bp intervals (Simpson and Whitlock 1976, Lutter 1977). This basic idea of DNA wrapping around the histone octamer was confirmed with the first low resolution atomic structure of the nucleosome which described histones adopting disc like conformations which are organised within the nucleosomal DNA repeat (Richmond, Finch et al. 1984). Continued structural studies yielded a 3.2 Å resolution model of the histone octamer as a left handed protein super helix in which histones adopt a conserved histone fold motif (Arents, Burlingame et al. 1991). Subsequently a series of atomic resolution structures of the nucleosome core particle have described the full range of interactions that form the nucleosome core particle (NCP) (Luger, Mäder et al. 1997, Richmond and Davey 2003).

Histones H2A-H2B and H3-H4 directly interact through association of their histone folds which each consist of three α -helices joined by the L1 and L2 loops, thus forming an $\alpha 1$ -L1- $\alpha 2$ -L2- $\alpha 3$ organisation. Interaction of histone pairs is mediated through organisation of histone folds along a pseudo-twofold symmetry. The axis of symmetry lies along the 8 turn central $\alpha 2$ helices, forming the “handshake” interaction. The (H3-H4)₂ tetramer is formed through interaction of two copies of H3 in which a 4 helix bundle involving $\alpha 2$ and $\alpha 3$ helices is formed. Completion of the octamer is achieved

through encapsulation of the core $(\text{H3-H4})_2$ tetramer by two H2A-H2B dimers. This interaction also occurs through two more four helix bundles (Luger, Mäder et al. 1997).

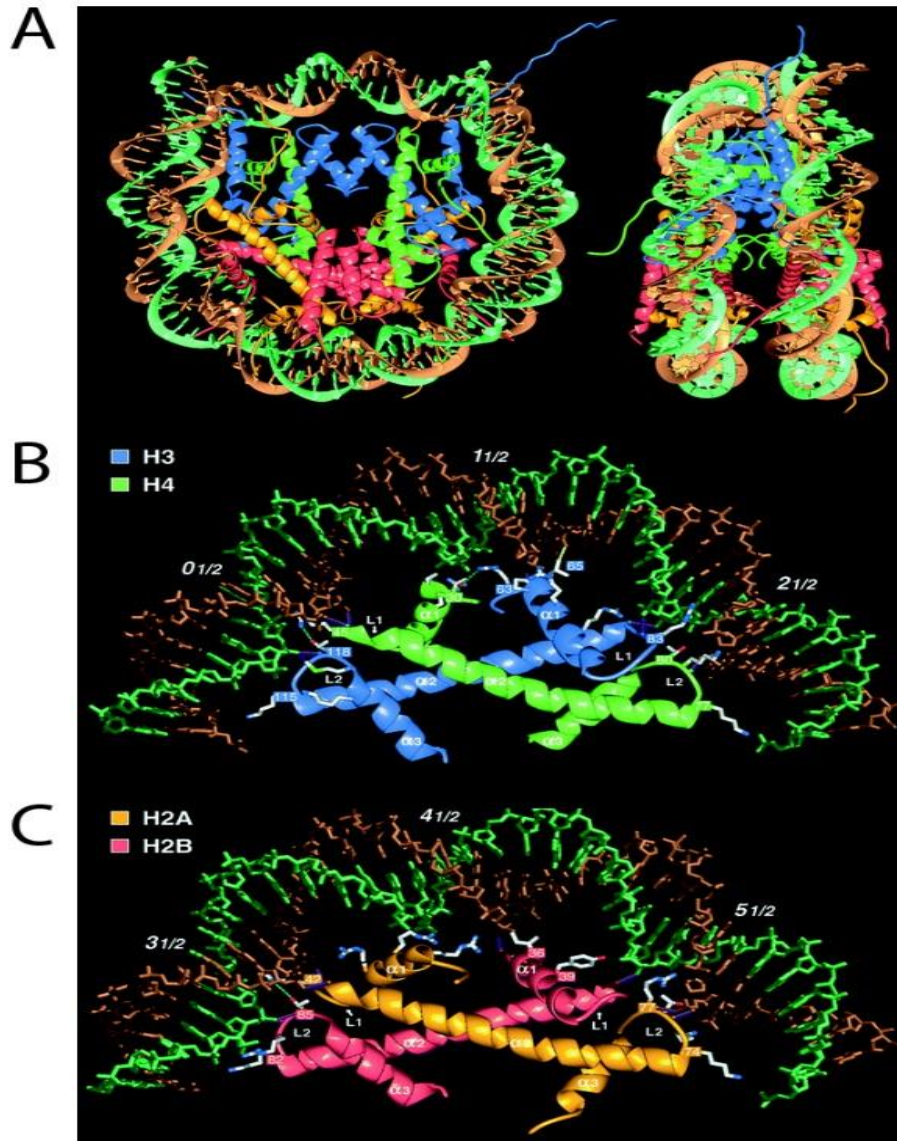


Figure 1-1 The atomic resolution crystal structure of the nucleosome core particle. A) The NCP displayed in two orientations. Two copies of each of the histone H2A (orange), H2B (red), H3 (blue) and H4 (green) form the core protein component of the NCP, around which ~147 base pairs of DNA are wrapped in a left-handed superhelical fashion. B) A histone fold dimer of H3 (blue) and H4 (green) and C) H2A (orange) and H2B (red) showing interaction with a segment of DNA within the nucleosome. (Adapted from (Luger, Mäder et al. 1997).

Surrounding the octamer is 1.65 turns of 146 bp nucleosomal DNA in a near two fold symmetry. 73bp and 72bp of DNA occur prior to and in advance of the central nucleotide, which is present at the nucleosome dyad. The rotational positioning of the nucleosomal DNA with respect to the dyad is described in terms of the super helical location (SHL), where the major groove of the DNA faces the octamer. SHL0 occurs at the dyad with SHL1-7 occurring at successive 10 bp DNA turns for the 1st 73 bp of the nucleosome. Subsequent major grooves after the dyad occur at SHL-1 to SHL-7 in the later 72 bp of nucleosomal DNA.

DNA wrapping around the octamer occurs independently of the DNA bases which allows chromatin formation in any genomic circumstance. Instead DNA contacts are mediated through hydrogen bonds between histone pairs and the DNA backbone. The histone-fold DNA-binding sites can be divided into two types. The first, or $\alpha 1\alpha 1$ site, uses both $\alpha 1$ helices of interacting histones to bind two DNA backbone segments at the centre of the bound DNA stretch. The second, or L1L2 sites are formed from juxtapositioned L1 and L2 loops and termini of the $\alpha 2$ helices at each end of the histone-pair (illustrated in figure 1.1B-C).

Arginine side chains located within the histone fold enter the minor groove 10 of the 14 times that it faces the histone octamer (Luger, Mäder et al. 1997, Richmond and Davey 2003). In addition H3 and H2B have extra α helical tails at the N' and C' termini respectfully which protrude into channels of the DNA superhelix. The locations of these helices have particular relevance with respect to post translational modifications of nucleosomes and nucleosome stability.

1.3 Genome-wide primary level chromatin architecture

1.3.1 Classical views of nucleosome organisation

The traditional method for determining nucleosome positions with respect to DNA sequence *in vivo* was indirect end-labelling (Clark 2010). This approach relies upon the inaccessibility of nucleosomal DNA to nuclease digestion. Classical techniques typically assayed DNA digestion as a measure of histone depletion (Weintraub and Groudine 1976, Wu 1980) using the endonuclease DNaseI. DNA fragments recovered post nuclease digestion are probed by southern blot to determine to locations of nucleosome protected DNA fragments at specific loci.

Latterly this approach was applied to the endo-exonuclease micrococcal nuclease (MNase) (Thoma, Bergman et al. 1984). Digestion of chromatin by MNase initially involves endonucleolytic cleavage of the linker DNA between nucleosomes. As digestion proceeds, the average number of nucleosomes per chromatin fragment decreases. The average fragment size for a given number of nucleosomes also decreases because the trimming activity of MNase shortens the cut linker at each end of the nucleosomal oligomer. Continued exo-nuclease activity removes residual linker DNA and halts at the nucleosome core particle (Hewish and Burgoyne 1973, Clark 2010).

Initial characterisation of primary level chromatin organisation suggested that nucleosomes were randomly positioned relative to the underlying DNA sequence of single copy genes (Prunell and Kornberg 1978). However it became clear that nucleosomes occupied defined positions at specifically tested loci, notably the 5' end of genes (Wu 1980, Bryan, Hofstetter et al. 1981, Samal, Worcel et al. 1981).

Underlying DNA sequence characteristics were heavily investigated as a factor that could define the observed positioning of nucleosomes. Although the histone octamer does not make contacts with DNA bases, nucleosome assembly will be facilitated by DNA sequences with the structural properties to accommodate wrapping around the octamer. Seminal studies by Travers and colleagues (Drew and Travers 1985, Satchwell, Drew et al. 1986) identified that nucleosomes from chicken erythrocytes contained AAA/TTT and AAT/TTA repeats rotationally aligned so that their minor groove faced toward the nucleosome core, whereas GGC/CGG and AGC/TCG repeats aligned such that their minor groove face away from the nucleosome core. This preferential positioning arises from the more malleable minor groove that AT dinucleotides create, allowing a more intimate binding with the histone octamer surface (Olson and Zhurkin 2011). That AT dinucleotide minor grooves are narrower also facilitates favourable interaction with arginines in the histone proteins (Rohs, West et al. 2009).

In addition to noting that phased AT sequences favoured DNA bending and nucleosome assembly, Travers and colleagues (Drew and Travers 1985, Satchwell, Drew et al. 1986) noted that PolyA sequences were disfavoured from the nucleosome core and were instead preferentially located at the ends of core DNA. Relative to random sequences, PolyA sequences are more rigid and might resist the distortion required to accommodate the histone octamer (Nelson, Finch et al. 1987). Functional significance for these structural considerations was provided by seminal *in vivo* observations that increasing the length of the poly(dA:dT) element at the *HIS3* promoter resulted in increased transcriptional activity and DNA accessibility (Iyer and Struhl 1995), an effect which is maintained on *in vitro* reconstituted chromatin (Sekinger, Moqtaderi et al. 2005).

These structural properties were further investigated by systematic evaluation of the preference for octamer assembly on genomic (Lowary and Widom 1997) and synthetic DNA sequences which facilitated (Lowary and Widom 1998) or occluded nucleosome formation (Cao, Widlund et al. 1998). TGGA repeats were found to be most refractory towards nucleosome formation (Cao, Widlund et al. 1998) whilst as expected AT dinucleotides at ~10bp repeats were found to selectively favour nucleosome assembly (Lowary and Widom 1998). However, that synthetic sequences far outperform the ability of native sequences to support nucleosome formation (Thastrom, Lowary et al. 1999) suggests the eukaryotic genome has not evolved to accommodate chromatin specifically. This observation supports initial findings that nucleosome formation potential is relatively equal across the genome (Lowary and Widom 1997, Thastrom, Lowary et al. 1999).

Classical observations also made clear a relationship between positioned nucleosomes and transcription. Seminal work by the Horz group characterised a *PHO5* promoter occluded by nucleosomes (Almer, Rudolph et al. 1986) under repressive conditions. Transcription activation is marked by a chromatin transition whereby promoter DNA becomes nuclease hypersensitive (Almer and Horz 1986) coupled with Pho2/Pho4 activator binding (Fascher, Schmitz et al. 1990). That Pho2/Pho4 were dependent for this chromatin reconfiguration to occur highlighted the role of trans-acting factors rather than DNA sequence in nucleosome organisation. Similarly, characterisation of the murine MMTV promoter showed that a positioned nucleosome was lost upon steroid induced gene activation (Richard-Foy and Hager 1987). Loss of this nucleosome was found to facilitate access of the transcription factor NFI at the promoter (Pina, Bruggemeier et al. 1990).

1.3.2 Genome-wide nucleosome organisation in the post genomics era

Over the past decade the advent of genomic technologies has led to an explosion in the numbers of studies mapping nucleosome positions genome-wide. Both classical and post genomic approaches rely on the inaccessibility of nucleosomal DNA to nuclease digestion, typically using DNaseI or MNase. Assay of nucleosome positioning genome-wide is possible using DNaseI digested chromatin (Hesselberth, Chen et al. 2009). However the approach is not favoured as it requires a wider spacing between nucleosomes in order to recognise and cleave genomic DNA, limiting its effectiveness to promoter regions. Instead modern techniques rely on MNase given its ability to digest shorter linker DNA sequences. While not the focus of this study, numerous alternative methodologies have been employed to assay genome-wide nucleosome organisation with converging outcomes (Rando and Winston 2012, Hughes and Rando 2014). That this and past studies rely upon MNase as a means of chromatin digestion, it is the principle area of attention.

Sequencing of MNase digested DNA allows identification nucleosome protected DNA fragments when the midpoints of these reads are mapped back to the reference genome. Pioneering studies using high resolution micro-arrays analysis showed that nucleosomes have distinct organisation and maintain specific positions (Yuan, Liu et al. 2005, Lee, Tillo et al. 2007, Schones, Cui et al. 2008). Progression in sequencing capabilities has allowed for high throughput, base pair resolution analysis of both DNA strands (paired end sequencing) using the Illumina platform. This allows for a more accurate determination of the DNA fragment midpoint which represents the nucleosome dyad location (Weiner, Hughes et al. 2010) in comparison to single end read analysis (Mavrich, Ioshikhes et al. 2008). Genomics allows analysis of two key characteristics of nucleosome organisation, positioning and occupancy. It is worth

mentioning here that there is a difference between the term nucleosome “occupancy” and “positioning”. Occupancy could be defined as a measure of histone or nucleosome density and it is typically measured in genomic scale using microarrays or deep sequencing (Kaplan, Moore et al. 2009, Pugh 2010). Positioning is a measure of the extent to which the population of nucleosomes resists deviation from its consensus location along the DNA and can be thought of in terms of a single reference point on the underlying DNA (Albert, Mavrich et al. 2007, Pugh 2010).

Initial characterisation of chromatin maps indicated that there was no obvious pattern of nucleosome organisation along a given length of the genome. However a remarkable organisation emerges when nucleosomal reads are aligned with respect to specific genetic elements and transcription factor binding sites (Yuan, Liu et al. 2005, Fu, Sinha et al. 2008, Koerber, Rhee et al. 2009, Berbenetz, Nislow et al. 2010). The nature of which is described in sections 1.3.3-1.3.4

1.3.3 Nucleosome organisation with respect to the transcription start site

Analysis of nucleosome density profiles has shown a specific organisation with respect to genes comprising of several conserved features which stretch from yeast to human chromatin (Yuan, Liu et al. 2005, Schones, Cui et al. 2008). Yeast promoters are characterised by a nucleosome depleted region (NDR) which is flanked by two defined nucleosomes. The NDR extends ~160-170 bp upstream of the transcription start site (TSS) which itself is located ~10 bp into the first coding region nucleosome, termed the “+1 nucleosome”. The +1 nucleosome is strongly positioned at the 5’ end of genes and is characterised by high read depth. The +1 is considered the best organised nucleosome. Regular spacing of nucleosomes across the gene body gradually dissipates towards the end of the gene as nucleosomes become more

delocalised (Mavrich, Ioshikhes et al. 2008). This is characterised by a reduction in the amplitude of the nucleosomal oscillation in the nucleosome density profile (see figure 1.2). Typical nucleosome spacing is every ~165 bp with 18 bp of linker DNA separating coding region nucleosomes (Lee, Tillo et al. 2007).

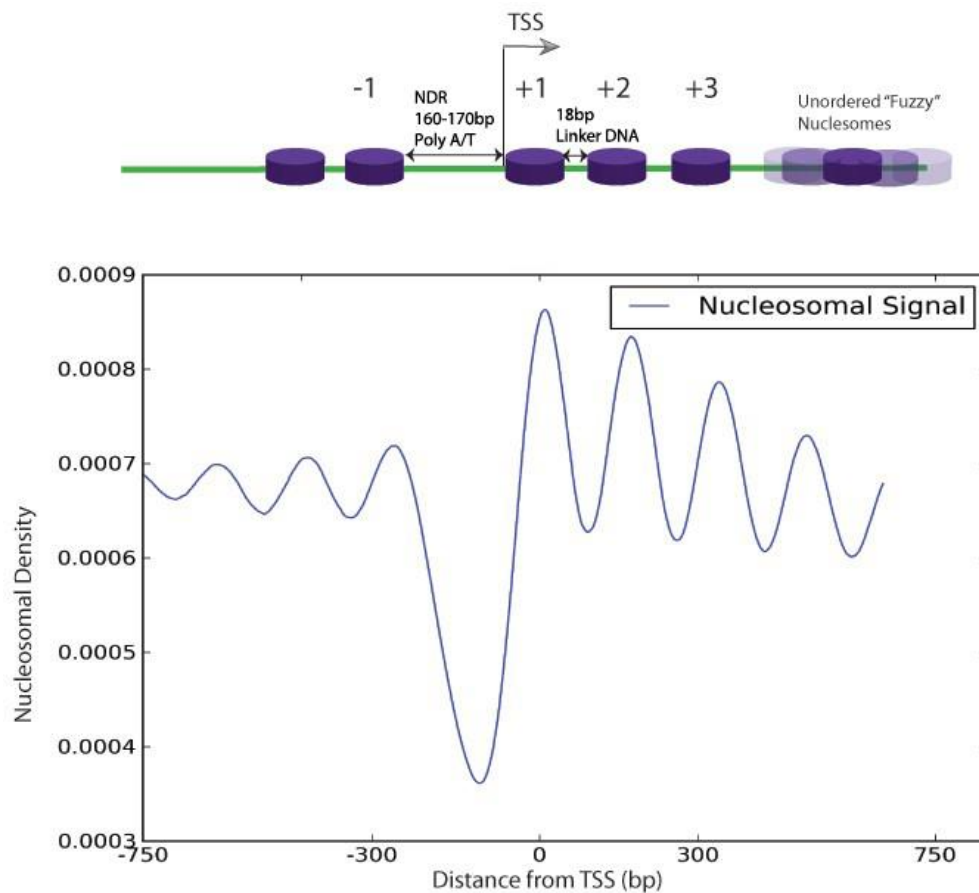


Figure 1-2 – Canonical organisation of nucleosomes with respect to transcription start sites. Plotting the frequency of nucleosomal dyads per base pair aligned to all yeast transcription start sites (TSS) shows periodic oscillations whereby peaks correspond to phased nucleosomes along the gene body while troughs correspond to linker DNA.

1.3.4 Nucleosome organisation with respect to origins of replication

In addition to transcription start sites, nucleosome organisation has been observed when aligned with respect to origins of replication. The origin recognition complex (ORC) denotes potentially active replication origins and binding in G₁ allows formation of the pre-replicative complex (Pre-RC). The *S. cerevisiae* ORC complex

recognizes the 11bp ARS (autonomously replicating sequence) consensus sequence (ACS) of competent origins where it is required to initiate origin firing each cell cycle (Sclafani and Holzen 2007). Nucleosome density aligned to the ACS displays an organisation not dissimilar to that of genic nucleosomes (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010). This consists of a narrower NDR of ~130bp which is centred ~36bp to the right of the ACS. Phased arrays of nucleosomes are arranged symmetrically with respect to the ACS. As observed for the TSS, this periodicity of positioning decays with distance from the ACS in the “+” and “-“ directions (see figure 1.3) (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010).

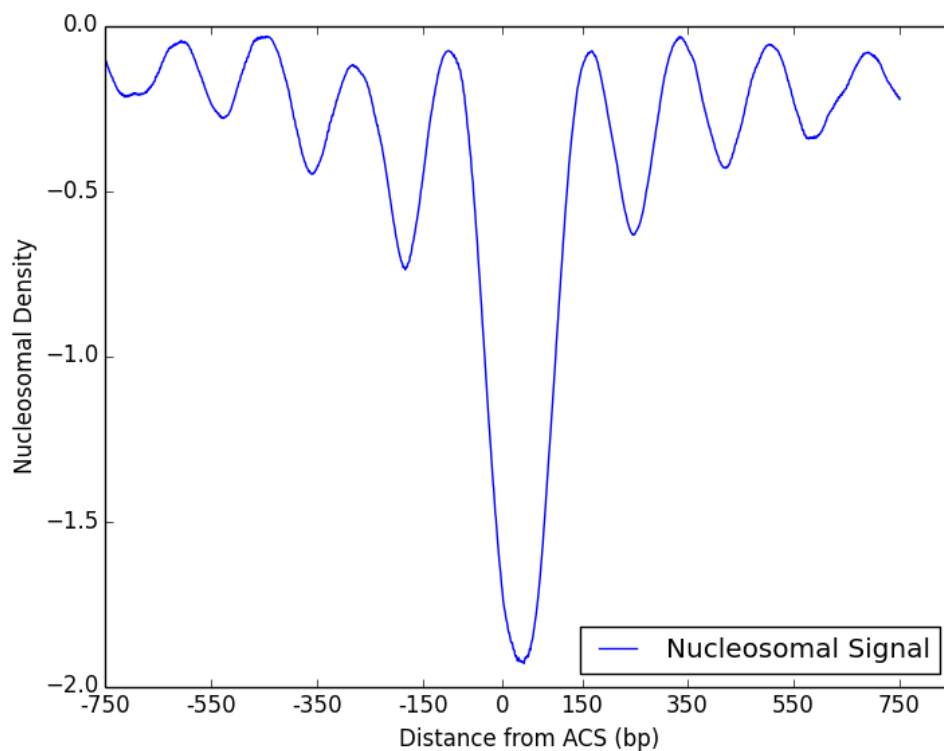


Figure 1-3 - Canonical organisation of nucleosomes with respect to the ACS. Plotting the frequency of nucleosomal dyads per base pair aligned to yeast ACS of selected replication origins (Berbenetz, Nislow et al. 2010) shows periodic oscillations emanating bi-directionally from the ACS. Peaks correspond to nucleosomes while troughs correspond to linker DNA. Figure adapted from (Berbenetz, Nislow et al. 2010).

The ORC complex appears crucial for maintaining nucleosome positioning and NDR width at origins (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010). These genomics studies compliment previous observations that ORC allows ARS1 firing through maintenance of nucleosome organisation (Lipford and Bell 2001). Given that the NDR surrounding the ACS is larger than that of the ORC binding footprint (Diffley, Cocker et al. 1994) it is likely that the ORC does not act alone and recruits additional factors to maintain NDR width and organise origin flanking nucleosomes.

1.4 Strategies for regulating nucleosome organisation

In addition to its role in DNA packaging, the correct arrangement of nucleosomes along a DNA sequence is also a regulatory mechanism that influences gene expression and other DNA-template dependent processes (Kamakaka and Thomas 1990, Simpson 1990, Durrin, Mann et al. 1992, Roth and Roth 2000, Lipford and Bell 2001). Over the past decade it has become clear that nucleosome organisation is determined by a dynamic combination of cis and trans determinants. The relative importance of cis and trans acting factors has been a source of some dispute as summarised in the following sections.

1.4.1 DNA sequence properties

Classical observations indicated DNA sequence plays a role in explaining nucleosome organisation (Satchwell, Drew et al. 1986), particularly at promoters (Iyer and Struhl 1995). Furthermore, identification of DNA sequences most complementary to nucleosome formation (Thastrom, Lowary et al. 1999) led many to evaluate if conserved nucleosome organisation observed in genome-wide nucleosome maps was

encoded by DNA sequence (Ioshikhes, Albert et al. 2006, Segal, Fondufe-Mittendorf et al. 2006).

Segal and colleagues identified DNA sequences which have strongly positioned nucleosomes *in vivo* and then use these sequences as a training set to compute common DNA sequence patterns in nucleosomal DNA segments. The identified nucleosomal DNA sequence motifs were then used to predict nucleosome positions across the genome and the predictions were compared to available nucleosome position maps (Segal, Fondufe-Mittendorf et al. 2006). Initially it was claimed that as much as half of nucleosome organisation could be described by sequence properties and that the +1 nucleosome was more likely than other nucleosome to be positioned by nucleosome favouring sequences (Segal, Fondufe-Mittendorf et al. 2006). A major limitation of this model is that predicted nucleosomes were only accurate within 35 bp of true *in vivo* locations. It is worth noting that random predictions would capture >30% of nucleosome positions within this distance. Furthermore the model failed to capture the characteristic nucleosome depletion at the NDR observed *in vivo*.

The failure of these models to describe nucleosome organisation led to experiments in which nucleosomes were assembled *in vitro* by salt dialysis using purified histones and genomic DNA (Kaplan, Moore et al. 2009). However, these failed recapitulate proper +1 nucleosome positioning (Zhang, Moqtaderi et al. 2009). In addition, *in vitro* chromatin reconstitution using limiting amounts of histones shows no sequence bias or preference in genic nucleosome location (Zhang, Wippo et al. 2011). This suggested more important roles for trans-acting factors in chromatin organisation, adding new complexity to a DNA encoded nucleosome organisation. Indeed, this was first observed when deletion of single or multiple ATP-dependent chromatin remodeling

enzymes leads to genome-wide loss in genic nucleosome organisation (Gkikopoulos, Schofield et al. 2011).

Instead DNA sequence effects appear strongest at poly (dA:dT) tracts where *in vitro* and *in vivo* datasets characterise nucleosome depleted regions at 5' promoters and 3' terminator regions (Kaplan, Moore et al. 2009). Intriguingly the poly (dA:dT) promoter DNA sequences observed in yeast promoters appear to vary across evolution. Promoter sequence features are similar between yeast and worm (Kaplan, Moore et al. 2009), although yeast species such as *S. pombe* have shorter poly (dA:dT) tracts and consequently narrower promoters (Lantermann, Straub et al. 2010). In contrast, many human promoters are characterised by high GC content, suggesting that higher eukaryotes have evolved different means of regulating promoters given that high GC content typically favours nucleosome formation (Hughes and Rando 2009, Valouev, Johnson et al. 2011).

Taken as a whole, observations from yeast indicate that DNA sequence primarily acts to deplete promoters of nucleosomes which in turn facilitates transcription. While regions of specific AT repeats at distinct intervals can facilitate strong nucleosome positioning, they are not a common feature of the yeast genome cannot describe positioning genome-wide (Hughes and Rando 2009).

1.4.2 Transcription factors

In vitro chromatin reconstitutions highlighted that nucleosome depletion at the NDR did not occur to the same extent as was observed *in vivo*, intimating the involvement of other factors (Kaplan, Moore et al. 2009). Indeed the same study identified that promoters bound by the general regulatory factors (GRF) Abf1, Reb1 and Rap1 *in vivo* showed no nucleosome depletion *in vitro*. This implicates a key role for these trans-acting factors given that *in vivo* analysis of their associated promoters highlighted a marked increase in nucleosome depletion in comparison to the genome-wide promoter average (Lee, Tillo et al. 2007). These observations are consistent with findings at single genes where Rap1 and Abf1 promote nucleosome depletion and facilitate transcription (Yarragudi, Miyake et al. 2004). Conditional deletion of Abf1 and Reb1 leads to increased promoter nucleosome occupancy coupled with disruption of promoter nucleosome positioning in distinct subsets of genes. This suggests these factors represent different mechanisms for establishing promoter nucleosome architecture (Hartley and Madhani 2009). This work was complimented by ChIP studies that showing that Reb1 and Rap1 bind at the -1 nucleosomes, potentially establishing the NDR by prevention of nucleosome encroachment into the promoter (Koerber, Rhee et al. 2009).

More recently insertion of exogenous yeast artificial chromosomes (YAC) in *S. cerevesiae* results in the YAC adopting host like chromatin organisation, with shifts in +1 position and formation of promoter like nucleosome organisation in non-coding regions. These sites were found to be recognised by *S. cerevesiae* transcription factors (TF) not present in the native YAC host (Hughes, Jin et al. 2012). These findings

implicate a vital yet poorly understood function of TF's in chromatin organisation genome-wide.

Mechanistically TF's may act as signals to recruit chromatin remodelers at certain promoters as observed with Reb1 and the RSC chromatin remodelling complex (Hartley and Madhani 2009) or perhaps the abundance of TF's in the nucleus (Newman, Ghaemmaghami et al. 2006) may allow them to outcompete other binding factors on newly synthesised DNA post replication to allow establishment of a barrier to nucleosome movement.

1.4.3 Histone variants

In addition to the four conserved core histone proteins, eukaryotes have evolved repertoires of less abundant histones known as variants. Initially identified as non-allelic genes (Franklin and Zweidler 1977), variants have different composition and genomic localisation in comparison to the core histones which impart specialised functions in processes such as gene regulation, chromosome segregation and DNA damage response (Talbert and Henikoff 2010).

While there is a large degree of heterogeneity in variants across eukaryotes, the H2A variant H2A.Z is conserved from yeast to vertebrates. H2A.Z is essential in mammals (Faast, Thonglairoam et al. 2001) yet the encoding gene *HTZ1* is dispensable in yeast (Santisteban, Kalashnikova et al. 2000) where many of its roles have been evaluated. The histone H2A.Z was first characterised in transcription regulation of the *PHO5* gene where it has a synthetic defect with the SWI/SNF chromatin remodeling complex (Santisteban, Kalashnikova et al. 2000). Initial genome-wide ChIP studies supported this finding further indicating that H2A.Z was primarily but not exclusively located

in promoter flanking nucleosomes (Li, Pattenden et al. 2005, Raisner, Hartley et al. 2005, Zhang, Roberts et al. 2005). Indeed some 5,000 +1 nucleosomes, of the ~60,000 total nucleosome population contain H2A.Z (Yen, Vinayachandran et al. 2013). Despite prevalence of H2A.Z at promoters, *htz1Δ* mutants retain canonical promoter architecture (Hartley and Madhani 2009).

Localisation of H2A.Z is dynamic and dependent on the seemingly antagonistic activities of the multi-subunit Swr1 and Ino80 chromatin remodeling complexes (reviewed in 1.4.5). Swr1 has long been known to incorporate H2A.Z/H2B dimers into nucleosomes at the expense of a canonical dimer (Mizuguchi, Shen et al. 2004). Swr1 activity is stimulated by the presence of canonical dimers and acts to replace both dimers in a stepwise fashion (Luk, Ranjan et al. 2010). On the other hand the Ino80 complex removes H2A.Z in a transcription dependent fashion and its deletion results in mis-localisation of H2A.Z (Papamichos-Chronakis, Watanabe et al. 2011). High resolution ChIP of individual Swr1 and Ino80 subunits suggests that both these factors bind to similar regions of the promoter allowing access to the +1 nucleosome. Their localisation has been shown to correlate with DNA binding factors Reb1 and Ies5/Nhp10 which are positioned at overlapping distances upstream from Swr1 and Ino80 respectfully (Yen, Vinayachandran et al. 2013). This finding compliments the previous observation that H2A.Z localisation to promoters is dependent on Reb1 binding (Hartley and Madhani 2009). That these two remodeling complexes have the same genomic localisation yet opposite activities would suggest that they are in constant state of competition or “futile cycle” which begs the question of what H2A.Z’s role is and why such dynamics would occur at the expense of ATP?

Peak H2A.Z incorporation occurs at the +1 nucleosome, which appears the most stringently positioned (Yuan, Liu et al. 2005), where it acts as a strong barrier to poise

transcription (Li, Pattenden et al. 2005, Zhang, Roberts et al. 2005). Perversely however, H2A.Z incorporation into the nucleosome imparts no improvement to the structural integrity of the nucleosome, in fact the opposite is suggested to occur (Suto, Clarkson et al. 2000, Zlatanova and Thakar 2008).

However, H2A.Z localisation at promoters co-indices with the regions of highest nucleosome turnover in the genome (Dion, Kaplan et al. 2007, Henikoff 2008). Given that H2A.Z has been observed to maintain heterochromatin boundary spreading (Meneghini, Wu et al. 2003) and is removed in a transcription dependent manner (Papamichos-Chronakis, Watanabe et al. 2011), H2A.Z may be involved in maintenance of chromatin states. Turnover of promoter proximal H2A.Z containing nucleosomes could re-set histone modifications that promote transcription after the event has occurred, preventing continued transcriptional activity.

H2A.Z is also implicated in the DNA damage response which involves another H2A variant, H2A.X. H2A.X is present in ~2-20% of nucleosomes in a non-specific manner (Weber and Henikoff 2014). H2A.X is phosphorylated at serine 139 by the ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) kinases which trigger the DNA damage response. This results in mass incorporation of H2A.X and DNA repair by non-homologous end joining or strand resection (Lans, Marteijn et al. 2012, Weber and Henikoff 2014). Yeast lack a specific H2A.X yet this role is compensated by an equivalent phosphorylation at serine 129 of H2A (termed γ -H2A) by members of the DNA damage response (Lydall and Weinert 1995, Downs, Lowndes et al. 2000). Again dynamics of H2A.X are dependent upon processing by chromatin remodelers that perform histone dimer exchange (Downs, Allard et al. 2004). The interplay between Ino80 and Swr1 has inferred a mechanism whereby γ -H2A is removed by Swr1 dependent incorporation of H2A.Z, which is subsequently

turned over for canonical H2A by Ino80 (Papamichos-Chronakis, Krebs et al. 2006). However it appears a lesser studied remodeler, Fun30 is primarily responsible for efficient strand resection after a double strand break (DSB) (Chen, Cui et al. 2012, Costelloe, Louge et al. 2012, Eapen, Sugawara et al. 2012). Fun30 appears to act by removing γ -H2A dimers from nucleosomes bound by the Rad9 checkpoint protein (Lydall and Weinert 1995, Lazzaro, Sapountzi et al. 2008). However the interplay between all three remodelers histone dimer exchange activities remains unresolved and warrants further investigation.

1.4.4 Histone modifications

Histones are adorned with multiple post translational modifications (PTM) which serve to alter histone:DNA interactions and more specifically act to mark nucleosomes with epitopes for biological processes (Kouzarides 2007, Bannister and Kouzarides 2011). Most modification takes place on the histone tails which protrude out from the nucleosomal core, although proteomic analysis has revealed less exposed sites within structured regions of the octamer are also modified (Mersfelder and Parthun 2006). A raft of residue specific modifications have been identified on lysine (acetylation, methylation, ubiquitination, sumoylation and crotonylation), arginine (methylation, citrullination, ribosylation), threonine (phosphorylation), serine (phosphorylation) and proline (isomerisation). Typically these modifications are not static entities as different groups of enzymes exist in a dynamic state of addition and removal of specific marks (Bannister and Kouzarides 2011, Tan, Luo et al. 2011). Acetylation, methylation and ubiquitination are the best studied modifications, hence detailed descriptions of these modifications are outlined below.

It has long been known that histones could be modified post translationally, initially at lysine residues. Pioneering experiments, incidentally both 50 years ago used ^{14}C carbon isoforms of methionine and acetate to follow their incorporation into histones in a translation independent manner, describing histone methylation and acetylation as the first observed histone modifications (Allfrey, Faulkner et al. 1964, Murray 1964). Acetylation is catalysed by a group of enzymes known as histone acetyl transferases (HAT) via the nucleophilic attack on the acetyl group of acetyl-CoA from the primary ϵ -amine of a lysine residue (Berndsen and Denu 2008). Chemically speaking, acetylation results in neutralisation of the positive charge on the modified lysine thus interrupting histone DNA contacts. This ties in with classical observations of acetylation resulting in a more open chromatin structure prone to nuclease digestion by DNAaseI digestion (Hebbes, Clayton et al. 1994) which was observed to facilitate transcription of the reporter gene α d-Globulin (Hebbes, Thorne et al. 1988). Acetylation is not a permanent mark and its dynamics on nucleosomes are maintained through removal by the histone deacetylase (HDAC) family of enzymes (Smith and Denu 2009).

Histone methylation mainly occurs on the side chains of lysines and arginines. Unlike acetylation, histone methylation does not alter the charge of the histone protein. There is an added level of complexity to bear in mind when considering this modification; lysines may be mono-, di- or tri-methylated, whereas arginines may be mono-methylated as well as symmetrically or asymmetrically di-methylated (Bannister and Kouzarides 2011). Focusing upon lysine methylation, histone lysine methyl transferases (HKMT) catalyse the transfer of a methyl group from S-adenosylmethionine (SAM) to a lysine's ϵ -amino group. The *Drosophila* enzyme SUV39H1 methylates H3K9 and was the first HKMT to be identified. It belongs to

the SET family of enzymes given that they contain a domain of the same name. These methylate N^o terminal tail lysines while the non-SET domain containing Dot1 methylates H3K79 in the globular core (van Leeuwen, Gafken et al. 2002). Modifications by these enzymes are specific to individual residues. The degree of methylation at a residue is also unique to individual methyl transferases (Tamaru, Zhang et al. 2003, Xiao, Jing et al. 2003). Previously considered infeasible due to the methyl marks stability, Lsd1 was identified as the first demethylase acting upon H3K4me_{1/2} (Shi, Lan et al. 2004). All of the other identified demethylases contain a jumonji domain (Jmj) domain (Whetstine, Nottke et al. 2006, Mosammaparast and Shi 2010). Like methylation, the reverse process also involves enzymes that are specific to particular residues and also to the extent of methylation (Bannister and Kouzarides 2011).

Unusually for a histone PTM, ubiquitination is a large 76 amino acid lysine modification. Modification requires the co-operation of E1-activating, E2-conjugating and E3-ligating enzymes (Hershko and Ciechanover 1998, Robzyk, Recht et al. 2000). A highly dynamic modification, it is removed by the deubiquitinating (DUB) family of enzymes (Schulze, Hentrich et al. 2011). Ubiquitination dynamics mark key events in the co-ordination of transcription and histone PTM's (outlined below).

Over the past decade there has been considerable interest in a “histone code” as a means of rationalising the occurrence of histone modifications and their associated biological processes (Strahl and Allis 2000). This hypothesis suggests that combinations of heritable modifications instruct downstream biological events and determine cell fate. The wealth of data generated in the post genomics era has suggested that the number of modifications are limited. Combinations of modifications can be grouped such that they describe different chromatin types with

specific functional properties (Filion, van Bommel et al. 2010). Interestingly, drug induced heterochromatin mediated gene silencing is maintained across multiple cell generations in stem cells (Hathaway, Bell et al. 2012). This gives evidence to the possibility that gene expression profiles could be heritable features.

Rapid nucleosomal turnover means that many modifications are often only present for the length of a transcription cycle (Dion, Kaplan et al. 2007, Deal, Henikoff et al. 2010). It is becoming apparent that histone modifications act to facilitate a highly dynamic cross talk between each other and different trans-acting factors functioning within the context of larger biological processes (Henikoff and Shilatifard 2011). This has been best studied in the context of transcription in budding yeast where a pathway of transient modifications occur along the gene body in concert with RNA polymerase II (RNAPII) at different stages of transcription elongation. This process tethers the activities of histone modifying enzymes, chaperones and chromatin remodelers. Disruption of this fine-tuned system leads to discrete alterations to the transcription programme and chromatin structure.

The status of RNA polymerase II (RNAPII) is communicated to chromatin via the C-terminal heptapeptide repeats (CTD) in its Rpb1 subunit. Initially serine 5 of the heptapeptide repeat is phosphorylated by the Cdk7 subunit of the transcription factor TFIIF which allows recruitment of RNA processing factors along with the accessory kinase Bur1 which phosphorylates the elongation factors Spt4/5. This promotes recruitment of the multi-subunit PAF (Paf1, Rtf1, Cdc73, Leo1 and Ctr9 proteins) complex (Li, Carey et al. 2007, Buratowski 2009, Jaehning 2010). The PAF complex facilitates recruitment of an E2/E3 ligase complex containing Rad6 and Bre1 which acts to monoubiquitylate histone H2B at Lys 123 (H2K123Ub) (Robzyk, Recht et al. 2000, Wood, Krogan et al. 2003, Xiao, Kao et al. 2005). H2BK123Ub is in turn

required for di- and tri- methylation of H3K4 by Set1 and H3K79 by Dot1. Both methyltransferases rely on the PAF complex for localisation (Dover, Schneider et al. 2002, Sun and Allis 2002). Progression of RNAPII to an elongation competent state occurs when Bur1 and Ctk1 kinases phosphorylate serine 2 of the Rpb1 CTD, a process which removes transcription termination factors associated with the polymerase (Buratowski 2009).

H3K4 methylation is recognised as a mark that prompts a destabilising acetylation of genic nucleosomes. The H3K4me3 mark may be recognized by histone acetyltransferases such as NuA3 (Taverna, Ilin et al. 2006) whilst the Sgf29 component of the SAGA complex recognises H3K4me2/3 to allow H3 acetylation by GCN5 (Govind, Zhang et al. 2007, Bian, Xu et al. 2011). Complementary to Set1, CTD phosphorylation at both serines 2 and 5 acts to target Set2 mediated methylation of H3K36me2/3 across the transcribed region, although this is biased towards the 3' end of genes (Li, Moazed et al. 2002, Krogan, Kim et al. 2003).

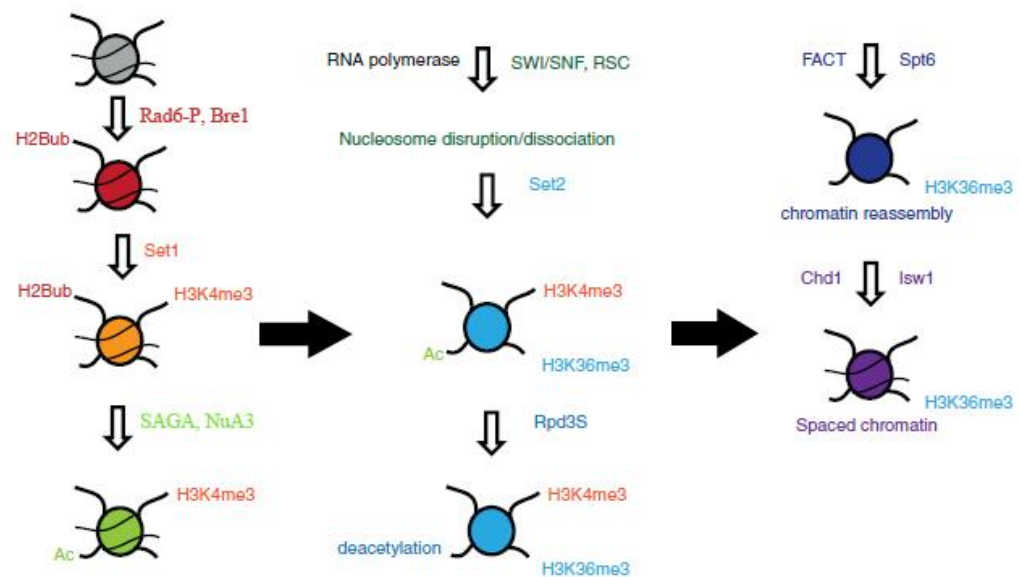


Figure 1-4 Systematic alterations to chromatin during stages of RNA polymerase II elongation. Adapted from (Owen-Hughes and Gkikopoulos 2012). The panels summarise the series of alterations to chromatin occurring during the course of transcription elongation that are discussed in the text. The left panel describes histone ubiquitination, methylation and acetylation that are coupled with RNA polymerase II becoming transcriptionally competent. The central panel describes RNA polymerase elongation and transcription coupled nucleosome disruption. Set2 methylation allows recruitment of Rpd3S to remove nucleosome destabilising acetylation. The right panel describes histone chaperone coupled chromatin reassembly and reorganisation of nucleosomes by chromatin remodelers on the genetic template.

While these modifications prompt further downstream modifications, they also encode their own erasure, thus preventing a perpetually open chromatin structure. The deubiquitinase (DUB) activity of SAGA subunit Upb8 removes H2B123Ub (Henry, Wyce et al. 2003, Lee, Florens et al. 2005, Wyce, Xiao et al. 2007). As H2B123Ub is needed for H3K4 methylation and Ctk1 mediated CTD phosphorylation at serine 2, this prevents re-initiation of the transcription cycle. Intriguingly, ubiquitinated nucleosomes exhibit highly organised chromatin with increased nucleosome occupancy (Batta, Zhang et al. 2011). Furthermore, through interaction with H2B123Ub, Spt16 of the FACT complex has been shown to promote faster polymerase elongation rates and histone deposition rates on the *GAL1* gene (Fleming,

Kao et al. 2008). Together these results suggest that ubiquitination serves to aid reassembly of nucleosomes post disruption by RNA polymerase II.

In addition to ubiquitination, nucleosome disrupting acetyl marks must also be removed to allow re-establishment of a non-transcribing chromatin state. This is achieved through the roles of the deacetylase complexes Rpd3S and Set3C which recognise methylated nucleosomes.

H3K36me3 is a mid to late occurring mark along the gene body and has important roles in reorganising chromatin post transcription. It targets Rpd3S to remove transcription coupled acetylation of genic nucleosomes. This is required to prevent excessive histone exchange that promotes spurious acetylation of H3K56 forming an open chromatin structure, resulting in cryptic and anti-sense transcription (Venkatesh, Smolle et al. 2012). A related study identified that chromatin remodelers Chd1 and Isw1b also co-localise with H3K36me3. They function to prevent the histone exchange that allows these transcription defects to occur (Smolle, Venkatesh et al. 2012).

The Set3C complex acts in a different manner and interacts with H3K4me2 which is proximal to the 5' end of genes. Deacetylation is performed by Set3C components Hos2 and Hst1 (Pijnappel, Schaft et al. 2001, Kim and Buratowski 2009). The main function of Set3C appears to be the regulation of transcription efficiency by prevention of cryptic transcription. The level of methylation (mono-, di- or tri-) at H3K4 decreases with distance from the promoter. Set3C has been observed to favourably bind H3K4me2 along this methylation gradient, deacetylating chromatin within these regions. These sites have been observed to act as promoters and in its role Set3C acts to prevent anti-sense/cryptic transcription from these regions. Set3C is thought to fine-

tune transcription by preventing anti-sense/cryptic transcription from outcompeting correct progression and kinetics of RNAPII along the DNA template (Kim, Xu et al. 2012).

1.4.5 ATP-dependent chromatin remodeling enzymes

Simple mixture of histones and genomic DNA via salt dialysis fails to recapture many features of *in vivo* like nucleosome organisation (Kaplan, Moore et al. 2009) suggesting a role for trans-acting factors. Support for this was gained from observations made from salt dialysis assembled chromatin treated with cell extracts (Zhang, Wippo et al. 2011). Interestingly, cell extracts were able to organise *in vivo* like chromatin organisation but in an ATP-dependent manner (Zhang, Wippo et al. 2011). The requirement of ATP for accurate chromatin organisation is driven by ATP-dependent chromatin remodelers. This is well demonstrated *in vivo* where removal of multiple remodeling enzymes results in complete loss of downstream genic nucleosome organisation (Gkikopoulos, Schofield et al. 2011, Hennig, Bendrin et al. 2012).

Dynamic chromatin states are facilitated by chromatin remodeling ATPases, whose roles account for a wide range of nucleosomal rearrangements. They are often referred to as Snf2 or SWI/SNF-related enzymes due to initial characterization of the yeast SWI/SNF complex as the first ATP-dependent chromatin-remodeling enzyme (Cote, Quinn et al. 1994). Snf2 proteins contain multiple amino acid sequence motifs that are conserved between the Snf2 protein and the superfamily 2 (SF2) grouping of helicase-related proteins (Gorbalenya, Koonin et al. 1988). Proteins related to Snf2 have since been identified within the genomes of all eukaryotes and based upon sequence

homology within the ATPase core can be assigned into 24 distinct subfamilies (Flaus, Martin et al. 2006).

Chromatin remodelers typically exist in multi-subunit complexes (notable exceptions include Fun30 and Chd1) where remodeler function is specified by ATPase and subunit composition (Clapier and Cairns 2009). Many subunits overlap between different complexes while subtle composition differences have been observed to result in functionally different remodeling activities (Vary, Gangaraju et al. 2003, Yen, Vinayachandran et al. 2012). In the most basic sense, chromatin remodelers use ATP to alter the DNA:histone binding interface. These remodeling outcomes can be grouped into 1) nucleosome sliding, 2) partial or complete nucleosome eviction and 3) alterations to octamer composition by histone dimer exchange (Clapier and Cairns 2009, Narlikar, Sundaramoorthy et al. 2013). However, how these enzymes function mechanistically is still poorly understood. Nucleosome sliding has been best characterised where recent observations were made during single molecule analysis of the Isw2 complex. FRET analysis indicated that DNA is initially pulled out from one side of the nucleosome causing a strain that results in DNA being pulled in from the other, resulting in translocation of the octamer along the DNA (Deindl, Hwang et al. 2013).

In vitro observations indicate that different remodelers facilitate different types of nucleosome translocation. Isw1a, Isw2, and Chd1 exhibit strong binding to nucleosomes carrying extranucleosomal DNA and tend to position nucleosomes centrally on short DNA fragments. In contrast Isw1b moves nucleosomes toward the end points of the DNA (Stockdale, Flaus et al. 2006). This specificity appears dependent upon remodeler binding to linker DNA which is facilitated by the ISWI family HAND-SANT-SLIDE domain (Gangaraju and Bartholomew 2007) and the

structurally related SANT-SLIDE domain in Chd1 (Ryan, Sundaramoorthy et al. 2011). Indeed both ISWI type remodelers and Chd1 show increased activity upon longer linker lengths (Whitehouse, Stockdale et al. 2003, Stockdale, Flaus et al. 2006). Furthermore, Chd1 repositions nucleosomes towards the end of DNA templates upon alteration to its SANT-SLIDE domain (McKnight, Jenkins et al. 2011).

In vivo, remodelers appear to override the intrinsic thermodynamic forces that aim to position nucleosomes along favourable stretches of DNA (Kaplan, Moore et al. 2009). Single gene analysis indicates that nucleosomes occupy positions dictated by DNA sequence in the absence of Isw2 (Whitehouse and Tsukiyama 2006). In contrast however, Snf2 is required to aid nucleosome removal from intrinsically unfavourable locations (Tolkunov, Zawadzki et al. 2011). Indeed, regions of nucleosome depletion not attained by salt based chromatin reconstitution require RSC activity for complete nucleosome depletion (Hartley and Madhani 2009).

Over the past decade, next generation sequencing has made it easier to address the roles of remodeling enzymes genome-wide. Initially Isw2 in yeast was found to regulate positioning at 5' and 3' ends of adjacent genes regulating cryptic transcription events (Whitehouse, Rando et al. 2007). Deletion and localization studies have demonstrated that specific remodelers tend to exhibit specific and directional effects with respect to transcription start sites (Gkikopoulos, Schofield et al. 2011, Yen, Vinayachandran et al. 2012). Isw2, Ino80, Isw1a (+1 nucleosome) along with RSC, SWI/SNF (+1, +2, +3) and Isw1b (+2, +3, +4) all appear to be enriched upon specific nucleosomes in the coding region. Consistent with earlier *in vitro* findings (Stockdale, Flaus et al. 2006), remodelers facilitate distinct directional shifts in nucleosome translocation. Ino80, Isw1b and SWI/SNF all appear to shift nucleosome distal to the

TSS *in vivo* while the opposite is the case for Isw2 and Isw1a (Yen, Vinayachandran et al. 2012).

Furthermore, groups of remodelers appear to function at distinct sets of genes cooperatively while other complexes such as RSC and SWI/SNF have little overlap with other remodeling complexes (Yen, Vinayachandran et al. 2012). These findings suggest that nucleosome organisation requirements vary on a gene to gene basis. While these studies describe collaborative nucleosome organisation by specific remodelers, a complete programme for organisation of nucleosomes on coding regions by remodelers is not yet known.

1.4.6 Histone chaperones

In primitive terms, histone chaperones are proteins that bind to histones in an ATP-independent manner that regulate correct nucleosome assembly. The importance of chaperones is illustrated by the seminal finding that simply mixing histones and DNA at low salt results in precipitation, requiring the chaperone nucleosplasin for stable transfer of histones onto DNA (Laskey, Honda et al. 1978). Chaperones are responsible for histone supply and demand which makes them key players in nucleosome assembly following DNA replication, DNA repair and gene transcription (Burgess and Zhang 2013). Chaperones share the common feature of being acidic which favours interaction with histones. Structurally however, histone chaperones are as diverse as the processes they facilitate. Chaperones mediate nucleosome assembly via replication-coupled (discussed in section 1.5) and replication-independent pathways.

Histone chaperones are thought to participate in distinct steps of nucleosome assembly. After synthesis in the cytosol histone proteins are imported to the nucleus. This role is fulfilled by chaperones such as Nap1 which help to shuttle newly synthesized H2A-H2B dimers from the cytoplasm to the nucleus (Mosammaparast, Ewart et al. 2002).

Chaperones also interact with the soluble pool of histones that is maintained for stress conditions. For example mammalian NASP, is thought to act as a histone reservoir and regulate histone supply during replication stress by protecting histones from degradation (Cook, Gurard-Levin et al. 2011). Furthermore, analysis of mammalian cytosol fractions shows that NASP interacts with histones H3 and H4 to facilitate dimer assembly prior to import into the nucleus, occupying a similar role to Nap1 (Campos, Fillingham et al. 2010)

Chaperones bind soluble histones and can modulate histone modifications that occur away from the exposed histone tails. RbAp46 and Asf1 directly regulate the enzymatic activity of histone modifying enzymes, serving to bridge interactions between histones and histone modifying enzymes (Parthun, Widom et al. 1996, Han, Zhou et al. 2007). RbAp46 is conserved from yeast to humans and promotes acetylation of histone H4 at lysine's 5 and 12 (H4K5,12ac) which is catalyzed by Hat1 (Parthun, Widom et al. 1996). Functionally, it appears that these modifications aid nuclear import. It has been observed that unmodified H3-H4 added exogenously to slime moulds is less favourably recovered in the nucleus in comparison to acetylated H4 (Ejlassi-Lassalette, Mocquard et al. 2011). Indeed, mammalian Hat1-RbAp46 mediated H4K5,12ac occurs in the cytoplasm and is an intermediate in the nuclear import of nascent H3-H4 dimers (Campos, Fillingham et al. 2010).

Asf1 is an evolutionary conserved chaperone that binds H3-H4 dimers along the H3 interface, preventing tetramerisation (English, Adkins et al. 2006). Asf1 is thought to supply H3-H4 dimers to the CAF-1 complex during replication (Li, Zhou et al. 2008, Malay, Umehara et al. 2008). In addition Asf1 is prerequisite for Rtt109 mediated acetylation of H3K56 (Driscoll, Hudson et al. 2007), a modification with replication dependent and independent roles (Rufiange, Jacques et al. 2007, Kaplan, Liu et al. 2008). Transient acetylation allows enhanced association of soluble H3-H4 with the chromatin assembly factor (CAF-1) complex and the Rtt106 histone chaperone which are thought to assemble (H3-H4)₂ tetramers onto the nascent DNA strands (Li, Zhou et al. 2008). Furthermore, Asf1 promoted H3K56 acetylation occurs at promoter proximal nucleosomes where it appears to contribute towards nucleosome turnover at these regions (Dion, Kaplan et al. 2007, Rufiange, Jacques et al. 2007, Kaplan, Liu et al. 2008).

Finally, histone chaperones are directly involved in the deposition of histones onto DNA during nucleosome assembly. The CAF-1 complex is considered primarily responsible for the deposition of H3-H4 onto DNA during replication (Stillman and Gluzman 1985, Stillman 1986). The complex comprises of the Cac1, Cac2 and Cac3 subunits also referred to as the p150, p60 and p48 respectively in higher eukaryotes. The Cac1 subunit of CAF-1 has been observed to associate with PCNA at the replication fork to allow histone deposition on nascent DNA (Shibahara and Stillman 1999). Yeast CAF-1 is thought to assemble to H3-H4 dimers into (H3-H4)₂ tetramers prior to deposition on DNA (Winkler, Zhou et al. 2012). Subsequent completion of the nucleosome with H2A-H2B dimers is likely performed by Nap1 or FACT complexes (Alabert and Groth 2012). Despite CAF-1 having a major role in H3-H4

deposition during S-phase, that all of its sub units are dispensable in yeast suggests that alternative, potentially redundant mechanisms for nucleosome assembly exist.

Chaperones also mediate replication-independent histone dynamics and have been implicated in determining genic nucleosome occupancy (Schwabish and Struhl 2004, Adkins and Tyler 2006, Schwabish and Struhl 2006). Genome-wide analysis of chromatin has revealed roles for the FACT complex and Spt6 in the maintenance of genic nucleosome organisation. Spt6 is a histone H3-H4 chaperone (Bortvin and Winston 1996) that binds nucleosomes (McDonald, Close et al. 2010) and is evolutionary conserved from yeast to human. Spt6 is localized throughout actively transcribed genes (Kim, Ahn et al. 2004, Mayer, Lidschreiber et al. 2010). Genome-wide analysis of chromatin in a yeast strain depleted of Spt6 shows that nucleosome occupancy across the coding region of genes is severely reduced, notably in the typically strong +1 nucleosome. These effects are magnified on highly transcribing genes suggesting that Spt6 functions in association with transcription and that Spt6 aids nucleosome reassembly following passage of RNA polymerase (Perales, Erickson et al. 2013).

FACT (facilitates chromatin transcription) is a histone chaperone critical for nucleosome reorganisation during replication, transcription, and DNA repair. Yeast FACT consists of two polypeptides, Spt16 and Pob3 which interact with a third, Nhp6 when bound to nucleosomes. FACT participates in reorganising nucleosomes through disrupting core histone-histone and histone-DNA contacts. Additionally, the FACT heterodimer possesses the ability to deposit H2A-H2B dimers and (H3-H4)₂ tetramers onto DNA (Winkler and Luger 2011). FACT is essential in yeast, however conditional deletion results in complete loss of nucleosome positioning and occupancy with respect to promoters along the coding region of genes (van Bakel, Tsui et al. 2013).

As observed in Spt6, this phenotype may result from a defect in reassembly in the wake of RNAPII progression along the gene. Indeed loss of the Spt16 subunit of FACT results in depleted levels of H2B and H3 in a transcription coupled manner at specifically tested genes (Jamai, Puglisi et al. 2009).

1.5 Chromatin Replication

Faithful replication of DNA and maintenance of its organization into chromatin are coupled during cell division. During S-phase, the chromatin landscape undergoes dramatic disruption as the entire genome is copied. Re-establishment of chromatin states is required in order that cell fate and identity are maintained (Alabert and Groth 2012). The model and practical evaluation of semi-conservative DNA replication provided a plain and aesthetically pleasing answer to the mechanism of genetic inheritance (Watson and Crick 1953, Meselson and Stahl 1958). However, the process of replicating chromatin states, including the transfer and deposition of histone proteins, distribution of histone modifications and translational positioning of nucleosomes at the replication fork have been less easily predicted and hence less is known about the process (Annunziato 2013).

Eukaryotic genome replication initiates at multiple sites, called replication origins. The budding yeast genome is replicated from hundreds of origins (Nieduszynski, Hiraga et al. 2007, Siow, Nieduszynska et al. 2012) and metazoan genomes from thousands of origins (Mechali, Yoshida et al. 2013). *Saccharomyces cerevisiae* replication origins are sequence defined and contain autonomously replicating sequences (ARS) which were initially shown to support plasmid replication (Stinchcomb, Struhl et al. 1979, Maundrell, Hutchison et al. 1988). Each origin

contains an essential 11bp sequence element called the ARS consensus sequence (ACS) (Nieduszynski, Hiraga et al. 2007, Siow, Nieduszynska et al. 2012).

Post mitosis, the ACS of replication origins are recognized by the multimeric origin recognition complex (ORC). During G₁ the MCM2–7 (minichromosome maintenance complex) is recruited to ORC bound locations in a Cdc6 and Cdt1 dependent manner forming the pre-replication complex (pre-RC) (Mechali, Yoshida et al. 2013). MCM2–7 loading allows origins to become ‘licensed’ and ready to be activated. Origins initiate replication or ‘fire’ via the sequential activity of two types of S-phase kinase. Together DDK (Dbf4-dependent kinase) and CDK’s (cyclin-dependent kinases) phosphorylate fork components allowing recruitment of Cdc45 and the GINS complex to activate the replicative helicase. The CMG complex (Cdc45–MCM2–7–GINS) is thought to constitute the core replicative helicase in eukaryotes (Bochman and Schwacha 2009). Unwinding of the double helix DNA at the replication fork allows continuous replication of the leading strand by DNA polymerase ϵ and semi-continuous lagging strand replication by DNA polymerase δ (Bell and Dutta 2002).

Nucleosomes are disrupted ahead of the approaching replication fork (Sogo, Stahl et al. 1986, Gasser, Koller et al. 1996), potentially due to collision with the replication helicases and new nucleosomes are assembled on the nascent strands. During S-phase appropriate numbers of nascent histones are produced to complement parental histones in organising an additional copy of the genome into chromatin (Alabert and Groth 2012). Initial studies of histone recycling indicated that parental histone octamers did not dissociate meaning that nucleosomes on the nascent DNA were fully composed of either parental or daughter histones (Annunziato 2013). However, studies employing careful labelling of amino acids and nascent DNA identified mixed or hybrid nucleosomes on nascent DNA where (H3-H4)₂ tetramers are conserved (ie, either

composed of either parental or nascent histones) (Jackson and Chalkley 1981, Annunziato, Schindler et al. 1982, Jackson 1988).

This was confirmed by modern mass spectrometry analysis of differentially mass labelled histones (Xu, Long et al. 2010). Splitting events that produce tetramers containing a mixture of new and old H3–H4 dimers occur only at low frequency. In yeast most old histone (H3–H4)₂ tetramers seem to be maintained in close vicinity to their original locus (Radman-Livaja, Verzijlbergen et al. 2011). The replication specific dynamics of histone H2A–H2B dimers are less clear, likely owing to their dynamic behaviour outside of S-phase (Dion, Kaplan et al. 2007, Deal, Henikoff et al. 2010).

There is a history of evidence suggesting that nascent chromatin has a distinct organisation. Radiolabelling with ³H thymidine for as little as 1 min showed that nascent chromatin was prone to digestion with DNaseI in comparison to bulk chromatin, a characteristic which persisted for ~15min (Seale 1975). Indeed, use of MNase in a similar fashion demonstrated preferable cleavage to mono nucleosomes on nascent chromatin (Hildebrand and Walters 1976). Electron microscopy of ssDNA bubbles representative of nucleosomes indicated that ahead of the replication fork nucleosomes are disrupted and are less stable on the nascent chromatin (Gasser, Koller et al. 1996). Furthermore using a replicating viral SV40 minichromosome, an asymmetry in distribution of the first nucleosomes on the leading and lagging nascent strands was observed whereby nucleosomes are placed further from the replication fork on the lagging strand (Cusick, Herman et al. 1981, Herman, DePamphilis et al. 1981, Sogo, Stahl et al. 1986). Given that nascent chromatin is more susceptible to digestion it is intriguing to find that nucleosomes are rapidly assembled on nascent chromatin after the passage of the replication fork (McKnight and Miller 1977, Sogo,

Stahl et al. 1986) as one may expect enhanced digestion due to loss of histone content. Furthermore, analysis of nucleosomes on nascent DNA at replication intermediates suggest that nucleosomes occupy the same positions as bulk (non-replicating) chromatin (Lucchini and Sogo 1994, Lucchini, Wellinger et al. 2001).

Recently, okazaki fragments have been mapped genome-wide through conditional depletion of the *S. cerevisiae* DNA ligase Cdc9 (Smith and Whitehouse 2012). Analysis of unligated nicks on the nascent strand suggests that ligation of okazaki fragments occurs within nucleosomal DNA at the dyad of known nucleosome positions which were found to determine the length of okazaki fragments. Perturbing elements of the CAF-1 complex which have been observed to alter replication dependent nucleosome assembly also disrupts okazaki fragment length suggesting that their positioning is defined by the presence of nucleosomes on the lagging strand. Given that okazaki fragments ligate at known nucleosome positions which dictate their length this suggests that nucleosomes maintain their positions after assembly on the nascent strand, agreeing with earlier findings (Lucchini and Sogo 1994, Lucchini, Wellinger et al. 2001).

However a major caveat with these studies is that they analyse a potentially static chromatin state. They either rely on capturing a long lasting replication intermediates (Lucchini, Wellinger et al. 2001) or conditionally deplete an essential enzyme for a protracted time course to allow maximal enrichment of okazaki fragments (Smith and Whitehouse 2012). In both instances, events which act to rapidly reorganise chromatin would not be detected. In this study we aim to develop methods of mapping and assessing the organisation of nascent chromatin genome-wide at selected stages of DNA replication. Analysing chromatin during active replication may allow for identification of a truer nascent chromatin state

2 Materials and Methods

2.1 Yeast transformation

2.1.1 Deletion cassette PCR

Deletion inserts were made through amplification of a resistance gene marker from *pFA6a-natNT2* (CloNAT) and *pFA6a-hphNT1* (Hygromycin B) plasmids. The amplification product was flanked by 50bp of homology, up and downstream of the gene of interest allowing deletion by homologous recombination.

The following is the PCR recipe used for generating such constructs:

<i>Reagent</i>	<i>Amount (μl)</i>
<i>H₂O</i>	27.75
<i>Forward Primer (10 μM)</i>	1
<i>Reverse Primer (10 μM)</i>	1
<i>BSA (10.5 mg/ml)</i>	0.5
<i>dNTP mix (3 mM each)</i>	4
<i>Plasmid Cassette (10ng/μl)</i>	10
<i>Buffer 2</i>	5
<i>Expand DNA Polymerase</i>	0.75

Table 2-1 Reaction cocktail for PCR amplification of yeast deletion cassette

PCR was performed using Expand High Fidelity PCR System®, product no: 11732641001

This mixture was incubated in a thermocycler under the following conditions:

Temperature °C	Duration (min)	Number of Cycles
94	3	1
94	1	30
55	1	30
68	2	30
68	7	1

Table 2-2 Thermocycler conditions for PCR amplification of yeast deletion cassette

2.1.2 Primer pairs used for deletion insert

Bar1::Hygomycin deletion primers

Forward

ATCGCCTAAAATCATACCAAATAAAAAGAGTGTCTAGAAGGGTCATATACGTACGCTG
CAGGTCGAC

Reverse

ACTATATATTTGATATTTATATGCTATAAAGAAATTGTACTCCAGATTTTCATCGATGAATT
CGAGCTCG

Asf1::CloNAT deletion primers

Forward

TAAACAGCGTACTCTCCCTACCATCCAATTGAAACATAAGATATAGAAAAGCGGATCCCC
GGGTTAATTAA

Reverse

TAAAGTGTACCTCTCTTGCAGGTACCATTAATCTTATAACCCATAAATTCGAATTCGAGC
TCGTTTAAAC

Cac1::CloNAT deletion primers

Forward

TATGTTTTAGTGAACCTCAAGACAGAAGAGAATCGAAAGGAAAAGGGAAACGGATCCCC
GGGTTAATTAA

Reverse

TAAATAATCAGTTTATCTGTATGTTTCTATATACTAAAGATCCGTTCAAGGAATTCGAGC
TCGTTTAAAC

Fun30::CloNAT deletion primers

Forward

GTAAGGAACGTAAACAAGAAAAAGAGAGAAAATACGCTATAGTTGAAAAC
CGGATCCCCGGGTTAATTAA

Reverse

TGGTTTATTTTCTGCTTATCTATTTACTTTTTTACTATATTTTATTTATGAATTCGAGCTC
GTTTAAAC

2.1.3 Transformation protocol

Transformations of yeast using deletion inserts were carried out in accordance with (Knop, Siegers et al. 1999).

2.2 Alpha factor synchronisation

Synchronisation of Mat a type cells in G₁ approach was taken from (Burke, Dawson et al. 2000) by where *Δbar1* strains were treated with 50 ng/ml of alpha factor (zymo research) mating pheromone and grown for 2.5 hours and checked for shmoo formation using light microscopy.

2.3 Genomic DNA isolation

As per (Burke, Dawson et al. 2000).

2.4 EcorI digestion of genomic DNA

Genomic DNA isolated as per 2.4 was subject to Ecor1 restriction digestion using the following recipe:

<i>Reagent</i>	<i>Amount (μl)</i>
<i>Genomic DNA (500 ng/μl)</i>	20
<i>NEB Buffer (10x)</i>	5
<i>BSA (10 mg/ml)</i>	1
<i>EcoRI (10U/μl)</i>	1
<i>H₂O</i>	23

Table 2-3 reaction recipe for EcorI digestion of genomic DNA

Mixture was allowed to react for 1hr at 37°C prior to heat inactivation at 65°C for 30 min.

2.5 Chromatin digestion using MNase

The following is an adaptation of mechanical yeast disruption (Rizzo, Mieczkowski et al. 2011) and chromatin digestion (Kent and Mellor 1995) protocols.

- Spheroblast digestion buffer (SDB) recipe: 1M Sorbitol, 50mM NaCl, 10mM Tris-HCl (pH 7.5), 5mM MgCl₂, 1mM CaCl₂, 1mM 2-mercaptoethanol, 0.5mM Spermidine and 0.075% v/v Nonidet p40 alternative.
- MNase: Micrococcal Nuclease (Roche) is dissolved in 10mM Tris HCl (pH7.5), 10mM NaCl, 100µg/ml BSA to 15 units/µl.
- Stop solution: 5% SDS / 250mM EDTA

Cells were cross-linked by addition of formaldehyde to a final concentration of 1% v/v for 10 minutes at room temperature (RT). Crosslinking was quenched with addition of 2.5 M glycine to a final concentration of 0.125 M and cells were further incubated for another 5 min at RT. Crosslinked cells were washed 3x with ice cold TBS (20mM Tris pH 7.5, 120 mM NaCl)

2.5.1 Yeast disruption and chromatin digestion for stable isotope labelling/CsCl and EdU pulse labelling approaches

Adapted from (Rizzo, Mieczkowski et al. 2011), post washing with TBS cells were finally suspended in 500 µl of SDB. 1ml of 0.5mm glass bead were added to the suspension and subject to mechanical disruption (bead beating; 4x1 min sessions, 2 min on ice between sessions).

Protein concentration of the lysate was determined by Bradford (BSA standards) assay and 3mg of was adjusted to 1ml in SDB on ice. Unless otherwise indicated 15U (1 µl) of MNase was added for 10min at 37°C with shaking (800 rpm). The reaction was stopped by adding 100 µl of stop buffer and crosslinks were removed by incubation at

65°C overnight. Following RNase and proteinase K treatment, DNA was recovered by phenol:chloroform extraction.

2.5.2 Yeast disruption and chromatin digestion for synchronised EdU labelling approach

Yeast disruption and chromatin digestion as per (Kent and Mellor 1995) with the following minor differences. 1×10^9 cells were digested using zymolyase (0.6mg/ml) for 26min to remove the cell wall. Following washing with 700 µl of 1M sorbitol and gentle re-suspension in 1ml of SDB cells were treated with 50U of MNase for 10min at 37°C with shaking (800 rpm). The reaction was stopped by adding 100 µl of stop buffer and reverse cross linking at 65°C overnight. Following RNase and proteinase K treatment, DNA was recovered by phenol:chloroform separation.

2.6 FACS analysis of yeast DNA content

1ml of cells were harvested and spun down at 14,000 rpm for 10 sec. The cell pellet was resuspended in 70% v/v ethanol overnight. Cells were again spun down and approximately 1×10^6 cells were washed and resuspended in 1ml of 50mM Tris-HCl (pH 7.5). 10 µl of RNase (10 mg/ml) was added for 10 hours at 40°C with shaking (500 rpm). Cells were washed and resuspended in 500 µl of a buffer containing 211mM NaCl, 78mM MgCl₂ and 50mM Tris-HCl (pH 7.8). 55µl of propidium iodide (500ug/mL in H₂O stock) was added and left overnight at 4°C prior to flow cytometry.

2.7 Stable isotope labelling and CsCl gradient ultracentrifugation

2.7.1 Stable isoform labelling media

Differential mass labelling was initially performed using D-Glucose-¹³C₆ and Ammonium-¹⁵N sulphate (both Sigma-Aldrich) as carbon and nitrogen sources allowing heavy isotope labelling of DNA. This was performed in the following strain:

W303 MAT a ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 bar1Δ:: hphNT1 Gal⁺ psi⁺

Latterly, a stable isoform of glucose, D-glucose-¹³C₆,1,2,3,4,5,6,6-d₇ (Cambridge isotope laboratories) was used as a carbon source (in conjunction with ammonium chloride isoforms) that would allow increased density labelling when metabolised into DNA. This was performed in a strain transformed with plasmids encoding *ura3* and *ade2*, allowing uracil and adenine biosynthesis:

W303 MAT a trp1-1 can1-100 leu2-3,112 his3-11,15 Ura⁺ Ade⁺ bar1Δ:: hphNT1 Gal⁺ psi⁺

Cell growth and media preparation are identical only differing by the type of glucose isoform used.

Heavy growth medium

Adapted from (Raghuraman, Winzeler et al. 2001), recipe for 350ml of heavy medium, typical volume used per experiment :

1g - ¹³C Glucose*

0.1g – ¹⁵N Ammonium Sulphate

0.05g (each) - His/Trp/Leu/Ade[†]/Ura[†]

350ml - “N” medium (yeast nitrogen base w/o ammonium sulphate-1.61g/L, succinic acid-11.1g/L, NaOH-6.67g/L)

* Either D-Glucose-¹³C₆ or D-glucose-¹³C₆,1,2,3,4,5,6,6-d₇

[†] Not added when using strain competent for adenine and uracil biosynthesis

2.7.2 Cell growth and synchrony

Cells were grown in heavy media to an OD₆₆₀ of 0.66 at 30°C. The α -factor mating pheromone was added to a final concentration of 50ng/ml for 1 hour 30 min. Cell morphology was checked by light microscopy to ensure cells were in M or G₁ phase. Cells were collected and washed on cellulose filter membranes with 800 ml of warm YPAD. Cells were re-suspended in 350ml of YPAD containing 50ng/ml α -factor and grown for 60 min at 30°C. Cell morphology was again checked by light microscopy for shmoo formation representative of G₁ arrest. Cells were filter washed with 800 ml of YPAD and released into 350 ml of YPAD (isotopically light) S-phase medium at 23°C. Approximately 50ml of cells were collected at defined time points and treated with formaldehyde to allow fixation for subsequent chromatin digestion.

2.7.3 CsCl gradient ultracentrifugation

Centrifugation of genomic and restriction digested HH and LL DNA

A solution of CsCl (sigma) and T₁₀E₁₀₀ was made in accordance with (Martineau, Whyte et al. 2008). The differentially labelled DNA to be resolved was mixed with the CsCl solution and sealed in a 5.1ml ultracentrifugation tube (Beckman Coulter). Centrifugation (Vti 65.2 rotor) was performed at 44,100 rpm for 36 hrs and brought to rest with no brake applied.

Centrifugation of genomic and MNase digested HH and HL DNA

The following centrifugation procedure was employed in attempting to resolve differentially labelled DNA isolated from a Ura⁻ and Ade⁻ deficient strain where D-Glucose-¹³C₆ was used as the carbon source in the heavy medium.

A solution of CsCl (sigma) and T₁₀E₁₀₀ was made in accordance with (McCarroll and Fangman 1988). The differentially labelled DNA to be resolved was mixed with the

CsCl solution and sealed in a 5.1ml ultracentrifugation tube (Beckman Coulter). Centrifugation (Vti 65.2 rotor) was performed sequentially at 65,000 rpm for 18 hrs, 50,000 rpm for 18 hrs, 28,000 rpm for 3.5 hrs and brought to rest with no brake applied.

Optimised centrifugation of MNase digested HH and HL DNA

The following centrifugation procedure was employed in attempting to resolve differentially labelled DNA isolated from a Ura⁺ and Ade⁺ competent strain where D-glucose-¹³C_{6,1,2,3,4,5,6}-d₇ was used as the carbon source in the heavy medium.

Increased density of DNA labelling was achieved using a more dense (heavy) glucose isoform in a strain that allows greater efficiency in DNA labelling. However, this necessitated use of a CsCl gradient solution made to a higher starting concentration to allow resolution of differentially labelled DNA across an increased density range.

A solution of CsCl (sigma) and T₁₀E₁₀₀ was made to a starting density of 1.4 g/g (CsCl/T₁₀E₁₀₀). 90µl (in T₁₀E_{0.1}, pH 7.5) of MNase digested, differentially mass labelled DNA was mixed with 9.3564g of CsCl solution and sealed in a 5.1ml ultracentrifugation tube (Beckman Coulter). Centrifugation (Vti 65.2 rotor) was performed sequentially at 65,000 rpm for 50 hrs, 50,000 rpm for 18 hrs, 28,000 rpm for 3.5 hrs and brought to rest with the slow brake setting applied.

Fractionation and analysis of CsCl gradient

Ultracentrifugation tubes were fixed to a retort stand and pierced at the base and then top with a small bore needle. Mineral oil was pumped in the top of the ultracentrifugation tube forcing drop wise elution from the tube at a rate of ~400µl/min. 250 µl of CsCl gradient was collected per fraction allowing collection of ~20 fractions per gradient.

Gradient fractions were subsequently dialysed against water (50 ml) on a floating dialysis membrane (Millipore) for 60 min. Dialysed fractions were run on a 1.5% agarose gel in TBE and electrophoresed to allow resolution of the nucleosomal DNA bands. Quantification of DNA per lane was made using the Aida Imaging Software in 2D Densitometry mode. Quantified DNA levels were adjusted against a background signal attained from an empty gel lane.

2.8 Nascent DNA labelling using EdU thymidine analogue, biotin attachment and streptavidin pull down

EdU (5-ethynyl-2'-deoxyuridine) labelling of nascent DNA was performed in synchronous and asynchronous cultures using the following wild type strain:

Mat a GPD-TK (Thymidine Kinase)-URA 3 (5 copies) Padh1- hENT1:: Aur1C trp1-1 leu2-3,112 his3-11, 15, Ade⁺ bar1Δ:: hphNT1

Analysis of nascent chromatin in asynchronous cultures was performed in the following histone chaperone and chromatin remodeler strains:

Mat a GPD-TK (Thymidine Kinase)-URA 3 (5 copies) Padh1- hENT1:: Aur1C trp1-1 leu2-3,112 his3-11, 15, Ade⁺ bar1Δ:: hphNT1 asf1Δ:: natNT2

Mat a GPD-TK (Thymidine Kinase)-URA 3 (5 copies) Padh1- hENT1:: Aur1C trp1-1 leu2-3,112 his3-11, 15, Ade⁺ bar1Δ:: hphNT1 cac1Δ:: natNT2

Mat a GPD-TK (Thymidine Kinase)-URA 3 (5 copies) Padh1- hENT1:: Aur1C trp1-1 leu2-3,112 his3-11, 15, Ade⁺ bar1Δ:: hphNT1 fun30Δ:: natNT2

2.8.1 Continuous EdU labelling in synchronised cultures

Cultures were grown to an OD₆₆₀ of 0.66 at 30°C in YPAD and synchronised with α -factor as per 2.2. Cells were filter washed with YPAD and released into YPAD medium containing 50 μ M EdU at 23°C. Cells were harvested at defined time points and were fixed with formaldehyde for subsequent MNase digestion.

2.8.2 Pulse EdU labelling in asynchronous cultures

Cultures were grown to an OD₆₆₀ of 0.8 at 23°C in YPAD. EdU was added to a final concentration of 100 μ M EdU. Cells were harvested at defined time points and fixed with formaldehyde for subsequent MNase digestion.

2.9 Biotinylation of EdU labelled nascent DNA

Biotin azide was attached to EdU labelled DNA using the Click-iT® Nascent RNA Capture Kit (Invitrogen, C10365). EdU labelled DNA replaced EU labelled RNA in the protocol. Manufacturer's guidelines were followed with slight modifications to the existing protocol which can be found in full:

<http://tools.lifetechnologies.com/content/sfs/manuals/mp10365.pdf>

Separate recipes were prepared depending on whether the EdU labelled DNA was isolated from asynchronous or synchronised cultures.

<i>Order of addition</i>	Reaction component	Stock concentration	Final concentration	Volume (µl)
1	H ₂ O	-	-	Variable
2	Click-iT® EU buffer (Component B)	2x	1x	50 µl
3	CuSO ₄ (Component D)	25mM	2mM	8 µl
4	Biotin azide (Component C)	10mM	1mM	10 µl
5	EdU-DNA	-	10 µg total	Variable
6	Click-iT® reaction Buffer additive 1 (Component E)	400mM	10mM	2.5 µl
7	Click-iT® reaction Buffer additive 2 (Component F)	400mM	12mM	3 µl

Table 2-4 Reaction cocktail (100 µl total reaction volume) for EdU labelled DNA isolated from synchronised cultures

<i>Order of addition</i>	Reaction component	Stock concentration	Final concentration	Volume (µl)
1	H ₂ O	-	-	Variable
2	Click-iT® EU buffer (Component B)	2x	1x	75 µl
3	CuSO ₄ (Component D)	25mM	2mM	12 µl
4	Biotin azide (Component C)	10mM	0mM	10 µl
5	EdU-DNA	-	125 µg total	Variable
6	Click-iT® reaction Buffer additive 1 (Component E)	400mM	10mM	3.75 µl
7	Click-iT® reaction Buffer additive 2 (Component F)	400mM	12mM	4.5 µl

Table 2-5 Reaction cocktail (150 µl total reaction volume) for EdU labelled DNA isolated from asynchronously growing cultures

2.10 Magnetic streptavidin pulldown of biotinylated DNA

Isolation of biotinylated DNA was achieved using Dynabeads® MyOne™ Streptavidin T1 (Invitrogen). The pulldown protocol was adapted from the manufacturer's guidelines, which are described in full:

<https://www.lifetechnologies.com/order/catalog/product/65601>

- Binding and washing (B&W) Buffer (2X): 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl

20µl of magnetic beads were magnetically immobilised and re-suspended in 40µl of binding and wash buffer (2X). 40µl of DNA (in H₂O) was added to the reaction tube, mixed and allowed to react for 30 min with occasional gentle vortexing.

The magnetic beads were magnetically immobilised and washed twice with 200µl of 1X binding and wash buffer. The beads were resuspended in 50µl of 1% SDS and were placed at 70°C for 60 min. The beads were immobilised and the supernatant was collected. DNA was recovered through addition of 90µl of Agencourt AMPure XP beads (Beckman Coulter) and washed as per manufacturer's instructions. Recovered DNA was then used as input for library preparation for Illumina next generation sequencing.

2.11 Preparation of DNA libraries for paired end Illumina sequencing

DNA fragments were prepared for sequencing in four separate preparative steps. Initially DNA overhangs and nicks were repaired. Next, a single A-nucleotide is added to the 3' end of the DNA fragments which facilitates ligation of generic adapters. These adapters enable a PCR reaction to allow amplification of the fragment libraries that contain generic extensions. These sequences allow fragment binding to the

sequencing flow cell and contain sequence specificity enabling unique identification of DNA fragment reads from different samples post sequencing in silico.

DNA End Repair

DNA	25-50µl
10 x T4-ligase buffer (with ATP)(fermentas)	10µl
dNTPs (10mM)	4µl
T4 DNA pol (fermentas)	3µl
Klenow DNA pol (fermentas)	1µl
T4 PNK (fermentas)	3µl
H2O	up to 100µl

Incubate 30min. at +20°C.

Purify with 180µl of Agencourt Ampure XP Beads as per manufacturer's instructions, elute to 32µl of EB

A-nucleotide addition

DNA	32µl
Klenow buffer (fermentas)	5µl
dATP (10 mM)	1µl
Klenow exo- (fermentas)	3µl
H2O	9µl

Incubate 30min. at +37°C.

Purify with 90µl of Agencourt Ampure XP Beads as per manufacturer's instructions, elute to 20µl of EB

Adapter ligation

DNA	20µl
Adapter (0.005µM)	1µl
10 xT4 ligase buffer (with ATP)(fermentas)	5µl
T4 DNA ligase (fermentas)	1µl
H2O	up to 50µl

LIGATION: Incubate at +16°C overnight, then 30min. on room temperature.

Purify with 55µl of Agencourt Ampure XP Beads as per manufacturer's instructions, elute to 20µl of EB

Adapter Sequences

Top PE Adapter

ACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Bottom PE Adapter

P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG

PCR Amplification

DNA	0.75-20.5µl
5 x HF buffer (Finnzymes)	10ul
Phusion DNA polymerase (Finnzymes)	1,5ul
Oligos (5uM)(PE 1.0 and iPCR primers)	3+3ul
dNTPs (10mM)	2ul
Threhalose (1M)	10ul
H2O	0-19.75ul

PE 1.0 and iPCR primers were used in the PCR amplification reaction. PE1.0 is a standard primer while use of different iPCR primers allows multi-plexing of individual samples during sequencing. Shown below are the sequences for the primers PE1.0 and iPCR primer 1

PE 1.0

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCT
TCCGATCT

iPCR Primer 1

CAAGCAGAAGACGGCATACGAGATAACGTGATGAGATCGGTCTCGGCAT
TCCTGCTGAACCGCTCTTCCGATCT

PCR conditions

98°C	3min.	
98°C	40s	} 15,17,18 or 20 cycles*
65°C	30s	
72°C	1min	
72°C	10min.	

* 15 cycles were used for samples that were control samples, either asynchronous or synchronized bulk/total chromatin fragments.

*17 cycles were used for samples prepared from CsCl gradient ultracentrifugation.

*18 cycles were used for samples prepared from EdU labelled nascent DNA isolated from synchronized cultures.

*20 cycles were used for samples prepared from EdU labelled nascent DNA isolated from pulse labelled chromatin, isolated from asynchronous cultures.

The PCR reaction was cleaned using 90µl of Agencourt Ampure XP Beads. The amplified DNA was ran on a 1.5% agarose gel. A DNA band was cut approximately 125-500 bp band and a gel extraction using Qiagen Minelute kit was performed, eluting DNA to 11µl of EB.

2.12 Analysis of next generation sequencing data

Processing of raw and mapped sequencing data was performed in a linux environment. The Python scripting language was used to process data once converted to a BAM format. Visualisation of data was performed using the Python module matplotlib (Hunter 2007).

2.12.1 Raw data processing

Raw sequencing data, in FASTQ format was aligned to the reference *S. cerevesiae* genome and converted to a sequence alignment map file (SAM) using the Bowtie software package (Langmead, Trapnell et al. 2009). Bowtie was used to ensure that only paired sequencing reads that mapped to the reference genome with a maximum of 2 bp mismatches and a fragment length of 100-200bp were considered for further analysis. The resultant SAM file was compressed to a binary format using samtools (Li, Handsaker et al. 2009) to create a usable BAM file.

2.12.2 Nucleosomal density plot

Nucleosome density plot were created by counting the average number of nucleosomal dyads that occur per base with respect to a genetic element such as a TSS, ACS binding site or entire chromosome. The dyad location is considered to be the central base pair of two paired nucleosomal reads (Zhang and Pugh 2011). Pysam was used to fetch

reads at specific genomic locations from BAM files (<https://code.google.com/p/pysam/>).

Genomic co-ordinates for transcription start sites as well as transcription levels were taken from (Lee, Tillo et al. 2007). ARS consensus sequences (ACS) were taken from (Berbenetz, Nislow et al. 2010). Cell cycle specific genes were taken from (Rowicka, Kudlicki et al. 2007).

Replication timing profile data was taken from (Yabuki, Terashima et al. 2002) and (Muller, Hawkins et al. 2014).

2.12.3 Clustering and heatmap generation

Clustering was performed using Gene Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/>). Hierarchical clustering was performed in all cases with centred correlation and centroid linkage. The nucleosome density across all genes was visualised heatmaps and were generated using Java treeview software (Saldanha 2004).

2.13 Index of experiments performed in this study

This study employed two different methods of isolating nascent nucleosomal DNA in wild type *S. cerevesiae* strains. This was achieved in synchronised cultures via stable isotope labelling coupled with CsCl gradient ultracentrifugation and using the thymidine analogue EdU. Pulse labelling of nascent DNA was also performed by in asynchronous cultures. This pulse labelling approach was extended to three deletion mutant strains in *S. cerevesiae*. A summary of experiments is outlined below in table

2.6

Method of nascent DNA labelling	Time points taken for each method (min)	Figures where data appears in this study
Stable isotope labelling coupled with CsCl gradient ultracentrifugation	28, 33, 41, 60 and 90 min post release from α -factor arrest	3.7, 3.10, 4.1, 4.3
Continuous EdU labelling	35, 40 and 45 min post release from α -factor arrest	3.9, 3.10, 4.2, 4.4, 4.5, 4.7, 4.8, 4.9, 4.10
Pulse EdU labelling in wild type strain	5, 10 and 50 min post addition of EdU	5.2, 5.3, 5.4, 5.5
Pulse EdU labelling in <i>fun30</i>Δ mutant	5, 10, 50 and 90 min post addition of EdU	5.6, 5.7
Pulse EdU labelling in <i>asf1</i>Δ mutant	5, 10, 50 and 90 min post addition of EdU	5.8, 5.10
Pulse EdU labelling in <i>cac1</i>Δ mutant	2, 50 and 90 min post addition of EdU	5.9, 5.11

Table 2-6 – List of experiments performed in this study

3 Establishment and Validation of Approaches to Study Nascent Chromatin Architecture

3.1 Introduction

The advent of new genomic approaches has made it possible to study the localisation of nucleosomes across the genome (Zhang and Pugh 2011). The factors responsible for directing nucleosome positioning are the subject of considerable debate (Kaplan et al., 2010, Zhang et al., 2010). That nucleosomes are organised with respect to transcription start sites suggests that spacing is coupled to transcription (Radman-Livaja and Rando 2010). However, how nucleosomes are organised following replication prior to transcription remains unclear.

It is known that histones are transferred behind the replication fork onto both the leading and lagging strands (Cusick, Herman et al. 1981, Sogo, Stahl et al. 1986). This transfer occurs rapidly following the synthesis of newly replicated DNA (McKnight and Miller 1977). Newly replicated chromatin appears to be more prone to nuclease digestion than bulk chromatin (Seale 1975), the result of a potentially “immature” nascent chromatin state (DePamphilis and Wassarman 1980). This gives rise to a potentially immature nucleosome organisation following chromatin assembly. While studies have attempted to address this issue either using viral mini chromosomes (Cusick, Herman et al. 1981, Herman, DePamphilis et al. 1981) or at single loci over the range of 4 nucleosomes (Lucchini, Wellinger et al. 2001), a genome-wide analysis of nascent nucleosome organisation has not been performed. To this end we have developed methods of mapping and assessing the organisation of chromatin at selected stages of DNA replication. The first relies on density labelling of nascent DNA using

stable isotopes while the second is dependent on incorporation of a thymidine analogue into nascent DNA.

3.2 Results

3.2.1 Nascent chromatin isolation by stable isotope labelling and CsCl gradient ultracentrifugation

A classical method to study replication involves differential stable isotope labelling of DNA strands (Meselson and Stahl 1958). This involves heavy labelling of DNA by growth of cell cultures in media containing dense isoforms of glucose (containing ^{13}C) and ammonium sulphate (^{15}N). Prior to S-phase the cells are shifted to an isotopically light media containing light isoforms of glucose (^{12}C) and ammonium sulphate (^{14}N). This results in the incorporation of the lighter isoforms of carbon and nitrogen into nascent DNA as replication is semi-conservative (Meselson and Stahl 1958). Differential stable isotope labelling results in non-replicated and nascent DNA, referred to herein as HH (non-replicated) and HL (replicated) having a mass difference. The mass difference between parent and daughter DNA can be exposed by isopycnic centrifugation using CsCl gradients, famously demonstrated by Meselson and Stahl (Meselson and Stahl 1958).

A CsCl solution (containing DNA to be resolved) is made in the density range of the labelled DNA to be separated. Centrifugation at high speed ($\sim 265,000 \times g$) allows formation of a gradient of Cs^+ ions in the centrifugation tube which creates a range of densities. Each labelled DNA species will migrate to an equilibrative buoyant density in the gradient resulting in their physical separation (Birnie and Rickwood 1978). This technique has been more recently adapted to monitor DNA replication genome-wide

(Raghuraman, Winzeler et al. 2001). To date this approach has been used to separate differentially labelled DNA fragments with a size of several kilobases. Adaption of this technique to resolve shorter (~150 bp) nucleosomal DNA fragments would allow the nature of chromatin particles assembled immediately following replication to be studied (figure 3.1).

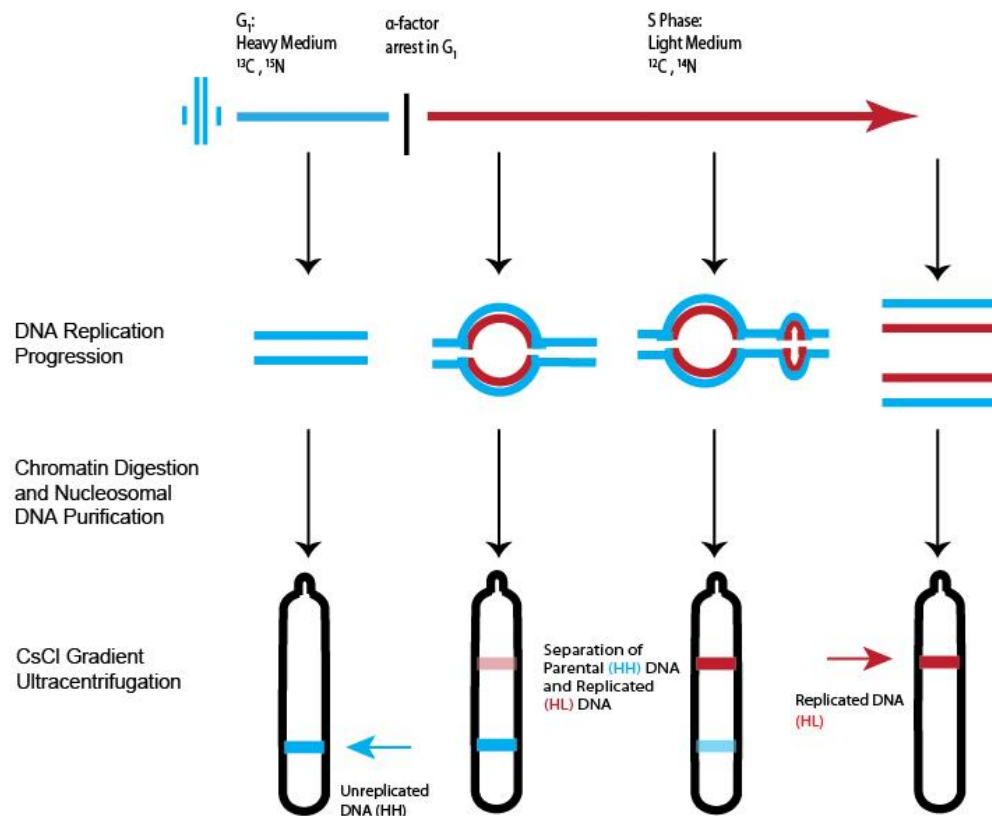


Figure 3-1 - Schematic for isolation of nascent nucleosomal DNA by stable isotope labelling and CsCl gradient ultracentrifugation.

3.2.2 CsCl gradients effectively resolve labelled and un-labelled DNA

Initially it was decided to test our ability to label DNA with stable isotopes and resolve differentially labelled species by CsCl gradient ultracentrifugation. To this end two yeast cultures of the same strain were grown in heavy (^{13}C , ^{15}N) and light (^{12}C , ^{14}N)

mediums to achieve fully heavy (HH) and light (LL) labelling of DNA. Isolated genomic and restriction digest DNA of each labelled species were mixed and spun in a CsCl gradient. Fractions were collected from the base (heavy end of gradient) of the ultracentrifugation tube and run on an agarose gel (figure 3.2B-C).

In resolving the heavy and light genomic DNA, separation between the HH (lane 11) and the LL (lane 15) labelled DNA is apparent (figure 3.2B). This separation is comparable with previous work pertaining to HH and LL separation (Neufeld, Vohra et al. 2007).

In order to assess the separation of a range of shorter fragments, the same approach was next applied to EcoRI digested genomic DNA (figure 3.2C). The HH digest material appears to form a band at fractions 9/10 while the LL digest material bands at fraction 13. Measurement of refractive index (RI) of the samples indicated that fraction 9 has an RI of 1.4046 and fraction 13 has an RI of 1.4003. This corresponds well with findings that HH (labelled with ^{13}C and ^{15}N) and LL DNA have RI's of 1.4050 and 1.4001 respectively (Birnie and Rickwood 1978, Neufeld, Vohra et al. 2007).

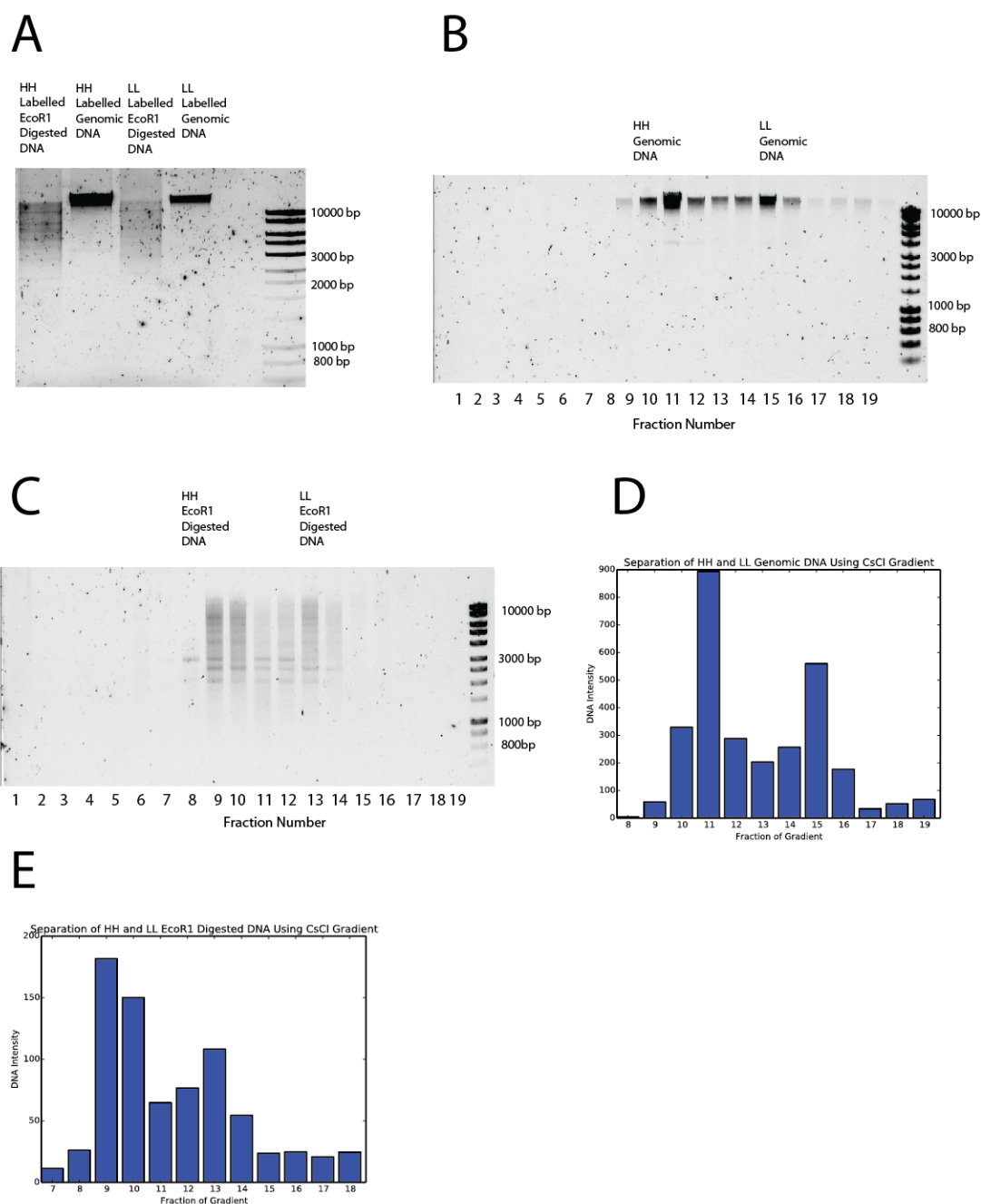


Figure 3-2 - Use of CsCl gradients to separate DNA fragments which have been labelled with stable isotopes.

A) A yeast culture was grown either in the presence (HH) or absence (LL) of heavy isoforms of carbon (^{13}C) and nitrogen (^{15}N). Genomic and EcoRI digested DNA was prepared from harvested cells and used as input for CsCl gradient ultracentrifugation. **B)** Isotopically labelled (HH) and unlabelled (LL) genomic DNA was separated via CsCl gradient prepared according to (Martineau, Whyte et al. 2008). The gradient was spun at 44,100 rpm for 36 hrs and brought to rest with no brake applied. Resultant fractions from the gradient were visualised on an agarose gel. Distinct DNA species occur at fractions 11 (HH) and 15 (LL). **C)** Isotopically labelled (HH) and unlabelled (LL) EcoRI digested DNA was separated via CsCl gradient prepared, run and fractionated as in B. Distinct DNA species occur at fraction 9 (HH) and 13 (LL). **D)** Normalised DNA levels for CsCl gradient fractions of separated HH and LL genomic DNA run on agarose gel. Two peaks occur at fractions 11 (HH) and 15 (LL) which are representative of the resolved DNA species. **E)** Normalised DNA levels for CsCl gradient fractions of separated HH and LL EcoRI digested DNA run on agarose gel. Peaks occur at fractions 9 (HH) and 13 (LL) which are representative of the separated DNA species.

3.2.3 Resolution of nascent and non-replicated DNA separation is diminished for nucleosomal sized DNA fragments

Application of stable isotope labelling and CsCl gradient ultracentrifugation techniques towards mapping of nascent mono-nucleosomes requires separation of HH and HL nucleosomal DNA which has been digested using micrococcal nuclease (MNase) (Hewish and Burgoyne 1973, Lohr and Van Holde 1975).

Considering the above approach we attempted to resolve genomic and MNase digested HH DNA and HL DNA (Figure 3.3). While it can be seen that separation of HH and HL genomic DNA is possible (Figure 3.3 B), it is striking that similarly labelled nucleosomal DNA species exhibit no obvious partitioning in the gradient (figure 3.3 D). Localisation of DNA to a fraction of the gradient appears dependent on fragment length. Progressively smaller fragments show pronounced smearing across Cs⁺ ion densities in the gradient. This is consistent with previous reports that smaller fragments do not separate well during CsCl gradient centrifugation (McCarroll and Fangman 1988).

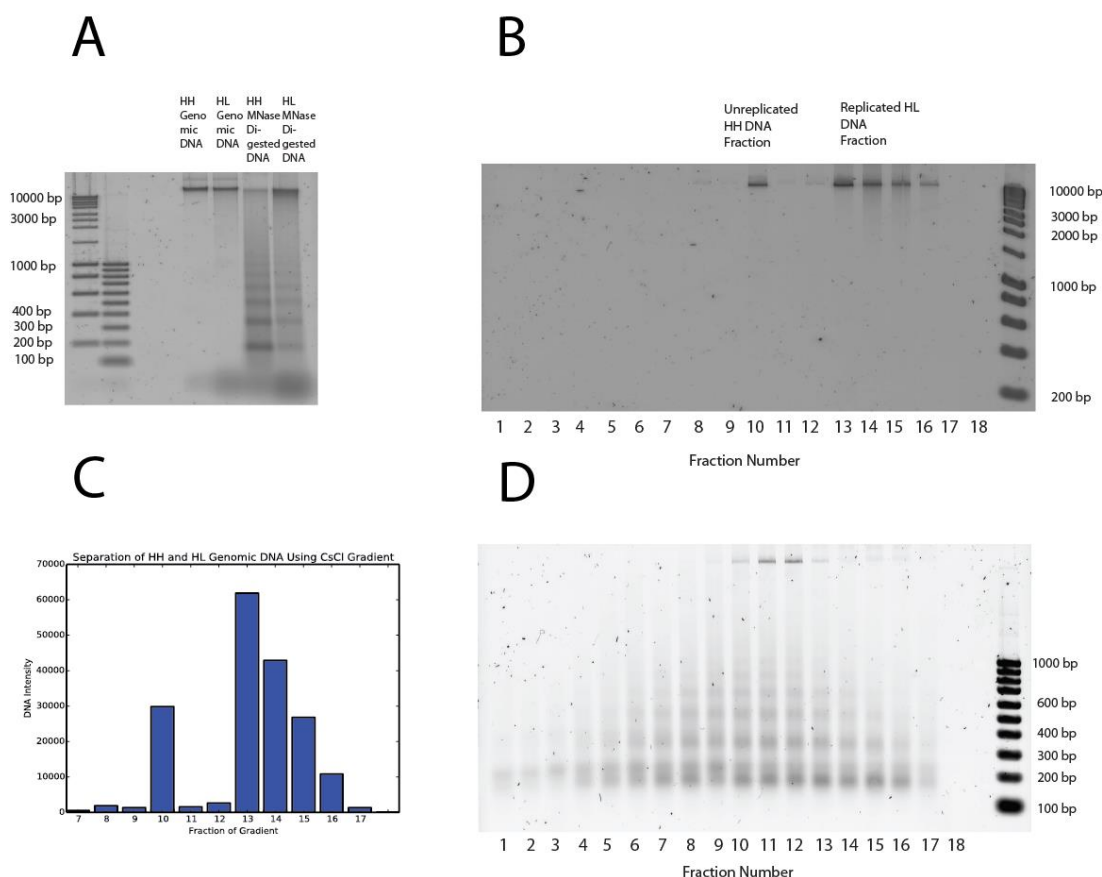


Figure 3-3 – Use of CsCl ultracentrifugation to revolve isotopically labelled nucleosomal DNA fragments. **A)** Yeast cultures were grown in heavy isoform growth medium to attain HH labelled DNA. Nascently replicated DNA was labelled by synchronising a culture growing in a heavy medium in G_1 using the yeast mating pheromone α -factor for 2.5hrs. Subsequently the yeast culture was released into an isotopically light medium for approximately one replicative cycle (~60min) resulting in HL labelled DNA. Harvested cultures were used to prepare labelled genomic DNA and MNase digested nucleosomal DNA fragments, whereby isolated total chromatin was subject to 15U MNase treatment for 10min at 37°C. **B)** Nascently replicated (HL) and non-replicated (HH) genomic DNA were separated via CsCl gradient prepared according to McCarroll and Fangman 1988. The gradient was spun at 65,000 rpm for 18 hrs, 50,000 rpm for 18 hrs and 28,000 rpm for 3.5 hrs. The gradient was brought to rest with no brake applied. Resultant fractions from the gradient were visualised on an agarose gel. Distinct non-replicated (HH) and replicated (HL) DNA species occur at fractions 10 and 13 respectively. **C)** Normalised DNA levels for CsCl gradient fractions in B), showing separation has occurred. **D)** Attempted separation of separately prepared nascent (HL) and non-replicated (HH) nucleosomal DNA fragments. Gradient was prepared according to McCarroll and Fangman 1988. Gradient was spun at 65,000 rpm for 18 hrs, 50,000 rpm for 18 hrs and 28,000 rpm for 3.5 hrs. The gradient was brought to rest with no brake applied.

3.2.4 Optimisation of nascent nucleosomal DNA isolation

Given the inability of previously established protocols to adequately resolve nascent and non-replicated DNA in the nucleosomal size range, we considered approaches to bolster the resolving power of the ultracentrifugation approach. Increasing the mass difference between HH and HL labelled DNA species would allow improved partitioning in a CsCl gradient. To ensure maximised labelling of non-replicated (HH) nucleotides with heavy isotopes, we considered transforming the previously utilised yeast strain with the *ura3* and *ade2* genes to allow biosynthesis of the nucleobases uracil and adenine respectively. These nucleobases are incorporated directly into deoxynucleotides during their synthesis. Biosynthesis of uracil and adenine through metabolism of glucose and ammonium sulphate isoforms should allow greater incorporation of ^{13}C and ^{15}N into synthesised DNA. This removes the need to supply growing yeast strains with exogenous, isotopically light uracil and adenine in the heavy growth medium.

Another approach to improve mass labelling involves increasing the number of heavy atoms incorporated into deoxynucleotides. To allow this an isoform of glucose, D-glucose- $^{13}\text{C}_6,1,2,3,4,5,6,6\text{-d}_7$ was used as a carbon source in the heavy growth media. All carbons in this isoform are substituted with the ^{13}C isotope and non-exchangeable hydrogen atoms are substituted with deuterium (figure 3.4). Additional hydrogen labelling would potentially result in an increased mass change of 13 to 18 Da per base pair between HH and HL labelled DNA (Sivakumar, Porter-Goff et al. 2004).

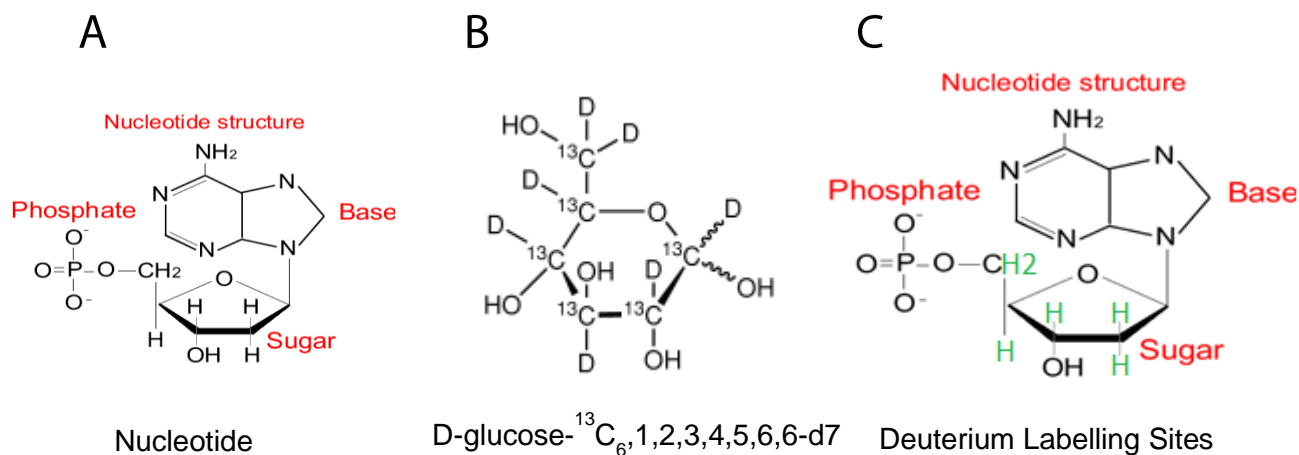


Figure 3-4 – Use of heavy glucose isoforms to density label non-exchangeable hydrogens on non-replicated DNA. **A)** Structure of an unlabelled nucleotide. **B)** Structure of glucose isoform D-glucose-¹³C₆,1,2,3,4,5,6,6-d₇. Heavy carbon isotopes are denoted by ¹³C while non-exchangeable hydrogens are replaced by deuterium (D). **C)** Structure of nucleotide indicating positions of hydrogens subject to deuterium labelling (green) when using deuterated glucose as a carbon source. Deuterium labelling only occurs on hydrogens bound to carbon.

These modifications to the existing protocol were tested by growth of cell cultures that could either produce uracil (Figure 3.5A) or both uracil and adenine (Figure 3.5B) biosynthetically in a medium containing heavy isoforms of ammonium sulphate and glucose (D-glucose-¹³C₆,1,2,3,4,5,6,6-d₇)(Figure 3.5). Cells were harvested at G₁ arrest (HH) and following passage through S-phase (HL) in a light medium. Isolated nucleosomal DNA of both labelled species were used as inputs for CsCl gradient ultracentrifugation. Unreplicated (HH) and nascent (HL) DNA were run in separate gradients along with a third gradient which contained a mixture of both labelled DNA species (HH and HL). Post centrifugation, collected gradient fractions were run on agarose gel and DNA content was quantified (Figure 3.5).

Considering first a yeast strain competent for uracil biosynthesis (Figure 3.5A), both non-replicated (HH) and nascent (HL) nucleosomal DNA have separate peak fractions (fractions 6 and 10 respectively) in the gradient when run in separately. Partial

separation of both DNA species is observed when attempting to separate them when mixed. The refractive index (R.I) measurement for the peak HH fraction was 1.4061, in comparison to an R.I of 1.4050 using the previous labelling strategy (Raghuraman, Winzeler et al. 2001). A greater R.I indicates that heavier labelling has been achieved which in turn contributes towards the slightly improved separation resolution of smaller, nucleosomal sized DNA fragments within the CsCl gradient.

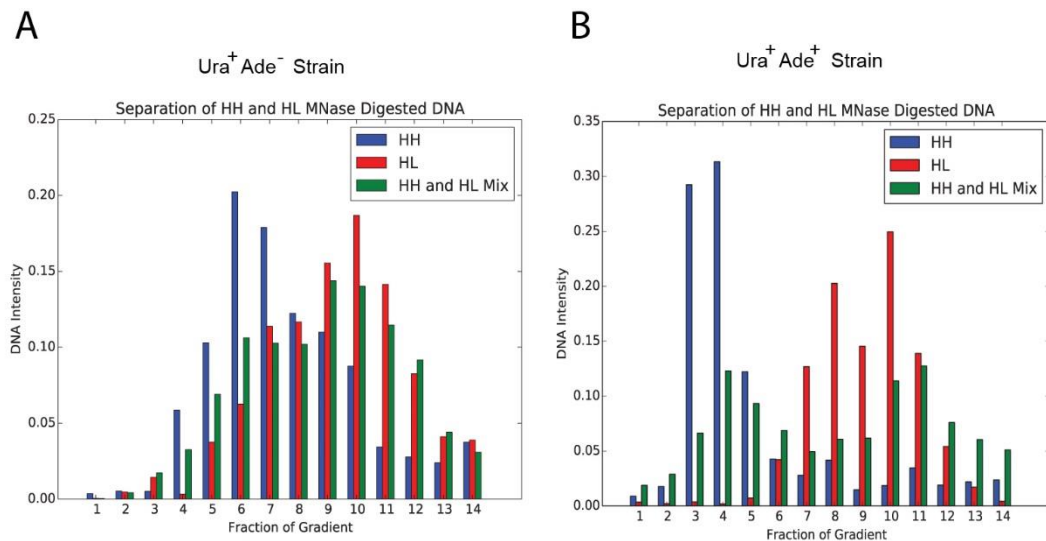


Figure 3-5 - Improving separation resolution of non-replicated and nascent nucleosomal DNA. A yeast strain capable of **A)** uracil and **B)** uracil and adenine biosynthesis was grown in a heavy medium containing deuterated ¹³C glucose as the sole carbon source. The culture was arrested at G₁ using α-factor and released into an isotopically light medium for one replication cycle (~60min). Harvested chromatin from G₁ and post S-phase cells was subject to 15U MNase treatment. Isolated nucleosomal DNA was used as input for CsCl gradient ultracentrifugation. G₁ arrested (HH), post S-phase (HL) and equal mixture of both (Mix) labelled DNA species were run on separate CsCl gradients prepared according to McCarroll and Fangman 1988. The gradient was spun at 65,000 rpm for 18 hrs, 50,000 rpm for 18 hrs and 28,000 rpm for 3.5 hrs. The gradient was brought to rest with slow brake applied. Fractions were collected from the gradient, run on agarose gels and DNA content per lane quantified. **A)** For a strain competent for uracil (but not adenine) biosynthesis, non-replicated and nascent nucleosomal DNA predominantly localise to fractions 6 (HH) and 10 (HL) when run in isolation. When these labelled DNA species are mixed, two partially resolved peaks in DNA content occur at these fractions. **B)** For a strain competent for uracil and adenine biosynthesis, non-replicated and nascent nucleosomal DNA predominantly localise to fractions 4 (HH) and 10 (HL) when run in isolation. When these labelled DNA species are mixed, two resolved peaks in DNA content occur at these fractions.

The improved separation achieved using a heavier glucose isoform in a strain with restored uracil biosynthesis prompted us to attempt further optimisation of DNA labelling by reintroducing the biosynthetic for pathway another nucleobase, adenine. This would allow complete incorporation of the stable carbon, nitrogen and hydrogen isotopes from the heavy medium into each deoxynucleotide. This is demonstrated in figure 3.5B where G₁ arrested (HH) and replicated (HL) nucleosomal DNA were spun individually and mixed together in three separate CsCl gradients. As before, individually labelled species occur in distinct fractions when centrifuged in isolation, HH DNA peaks at fraction 3 while the HL DNA peaks at fraction 10. When spun together, HH and HL DNA can be resolved as distinct species, appearing at the same locations of the gradient as each labelled DNA species when ran in isolation (Figure 3.5B). The HH peak fraction has a refractive index of 1.4072, greater than the value attained for the HH fraction in a strain requiring unlabelled exogenous adenine (1.4060). This further suggests improved mass labelling efficiency has allowed better isolation of nascent nucleosomal DNA fragments.

3.2.5 Preparation of nascent nucleosomal DNA for high throughput sequencing

Efforts to establish a method for isolation of nascent nucleosomal DNA by CsCl gradient ultracentrifugation relied on use of DNA from cells that were either G₁ arrested or had completed S-phase. However to map nucleosomes in dynamically replicating cultures, cells were harvested at specific time points post release from G₁ as shown in figure 3.6B. A slight modification to the CsCl gradient preparation was also introduced. The pre-spun gradient was made to a starting density of 1.4 g/g (in T₁₀E₁₀₀) as opposed to 1.28 g/g as used previously. Given the added efficiency in labelling capabilities, a gradient with a heavier starting density allows a more equal

separation of labelled DNA species across the full length of gradient tube during separation.

Analysis of agarose gels representing fractions of the gradient shows that DNA from G₁ arrested cells (HH) is resolved in fractions 6-12, peaking in fraction 9 (figure 3.6C). Mid S-phase DNA (HL) shows additional DNA in the less dense fractions 13-20 which corresponds to the nascently replicated DNA, peaking around fraction 16 (figure 3.6D). Fractions 9 and 16 were chosen as being representative of non-replicated (HH) and nascent (HL) chromatin for subsequent high throughput sequencing.

Using stable isotope labelling coupled with CsCl gradient ultracentrifugation, nascent and non-replicating nucleosomal DNA was isolated for G₁ arrested cells and defined S-phase time points post release from the α -factor arrest block. Purified mono-nucleosomal DNA from non-replicating and nascent CsCl gradient fractions was ligated to specific adapters and PCR amplified to prepare the DNA libraries for paired end Illumina sequencing (data not shown, see section 2.10 for full method).

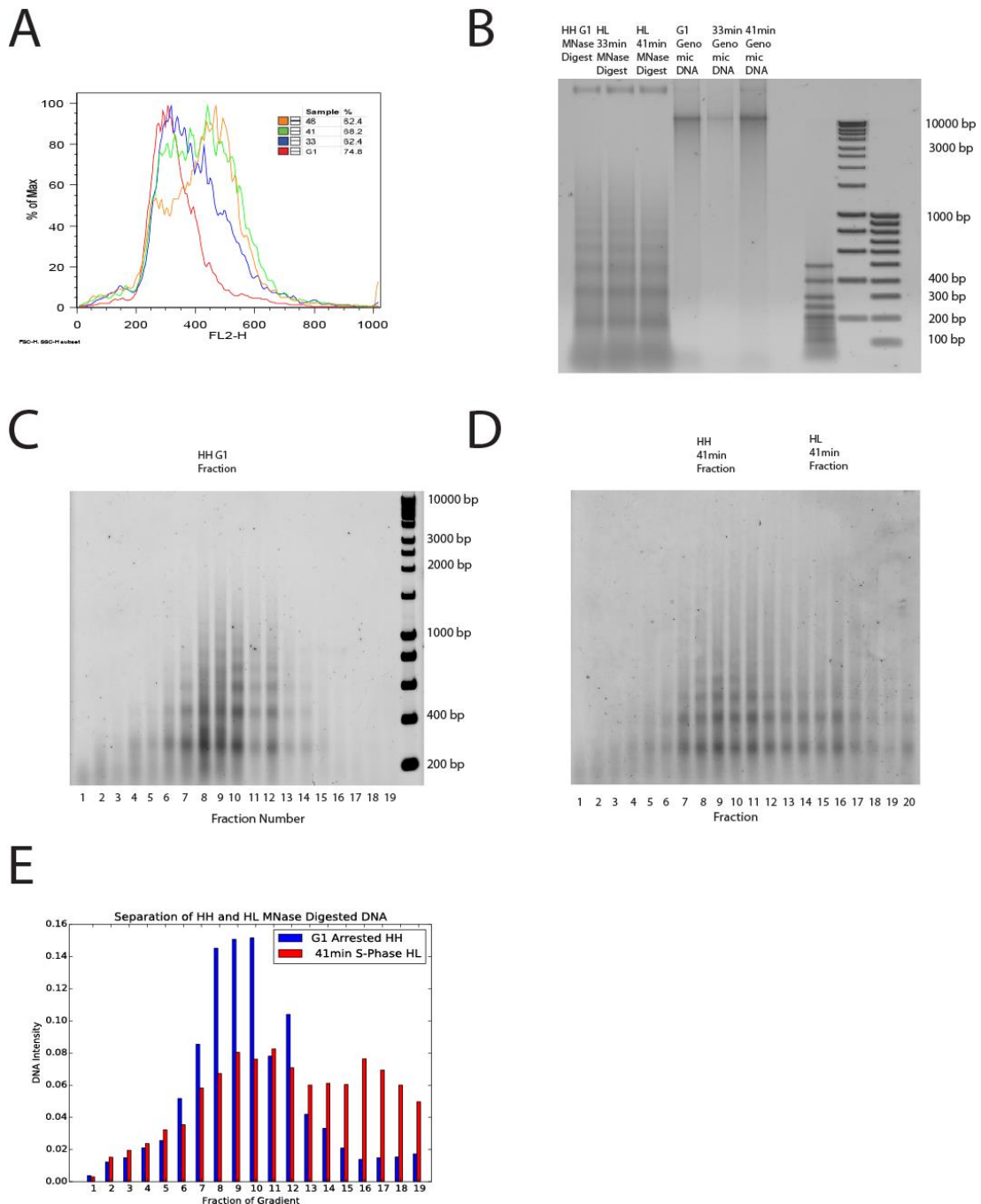


Figure 3-6 - Use of CsCl gradient to isolate replicating nucleosomal DNA fragments labelled with stable isotopes. A) FACS analysis of a yeast culture taken at defined time points post G₁ arrest in an isotopically light S-phase medium. It can be seen that the fluorescently labelled DNA content of the yeast culture increases over S-phase as the DNA fluorescence peak migrates from 1C (FL2-H 200) to 2C (FL2-H 400). **B)** MNase digest inputs for CsCl gradient analysis. Chromatin from yeast harvested at specified S-phase time points was exposed to 15U of MNase digestion for 10mins at 37°C. Controls for the reaction are present in subsequent lanes where chromatin was not subject to MNase **C)** Nucleosomal DNA isolated from a G₁ arrested yeast culture was spun on a CsCl gradient (1.4 g/g in T₁₀E₁₀₀ starting density) as in figure 3.5. Resultant DNA fractions were run on an agarose gel. A distinct DNA species occurs at fraction 9 (HH). **D)** Nucleosomal DNA isolated from a yeast culture 41 min post G₁ arrest was run on a CsCl gradient (as in C) and resultant DNA fractions were run on an agarose gel. It can be seen that two distinct species occur at fractions 9 (HH) and 16 (HL). **E)** Normalised DNA levels for each lane of the G₁ control and 41min S-phase time points. G₁ arrested nucleosomal DNA content peaks around fraction 9 in the gradient whilst two species of DNA are present at fractions 9 (non-replicated HH) and fraction 16 (Nascent HL) for a mid S-phase time point.

3.2.6 Validation of CsCl based isolation of nascent chromatin using genomics

Subsequent to high throughput sequencing of non-replicated and nascent nucleosomal DNA it was decided to investigate whether the isolated chromatin was replication specific. To this end we plotted the number nucleosomal dyads (midpoint of each nucleosomal fragment) as a function of base pair along yeast chromosome 13 (figure 3.7). For an early S-phase time point, 33min post release from G₁, the read depth of HL DNA tends to be variable across the length of the chromosome (figure 3.7A-B). In contrast the read depth of the non-replicated HH DNA remains evenly distributed across the chromosome (figure 3.7B). This suggests that localised regions of higher read depth across the chromosome correspond to newly replicated DNA synthesised from origins of replication while regions of low read depth have yet to be replicated due to the absence of active origins (Raghuraman, Winzeler et al. 2001, Yabuki, Terashima et al. 2002). Indeed, this appears to be case when comparing the profile of nascent read depth across the chromosome to previously annotated replication profiles (figure 3.7C) (Yabuki, Terashima et al. 2002). There is a clear overlap in terms of location and profile of mapped origins of replication to the HL read depth across the chromosome, indicating that regions of high read counts represent nascent nucleosomal DNA. In addition the HL read distributions compare favourably (Pearson correlation 0.862) to recent ‘Sort-seq’ data which analysed replication dynamics in asynchronous S-phase populations (Muller, Hawkins et al. 2014).

Completion of replication is observed when assessing replicated material from a late time point post release into S-phase (figure 3.7D-E). A more even distribution of nascent read depth across the chromosome indicates that replication has completed.

Collectively, these observations indicate that our isolated nucleosomal DNA is indeed replication specific.

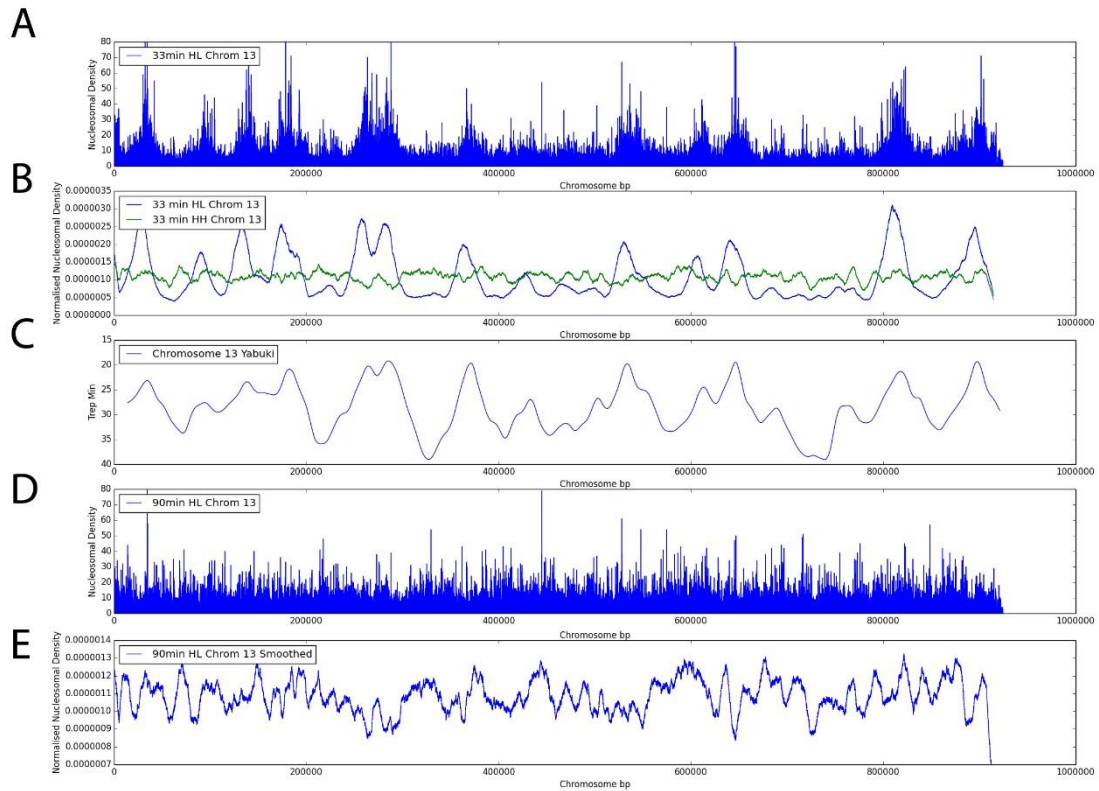


Figure 3-7 - Validation of nascent chromatin isolation by CsCl gradient using genomics. A) Total number of nascent nucleosomal reads per bp along chromosome 13 for an early S-phase time point, 33min post release from G₁ arrest. Distribution of nascent read enrichment along the chromosome is similar to replication profiles of previously annotated origins (C). **B)** Nucleosomal reads plotted as a 10,000bp moving average for replicating (HL) and unreplicated (HH) DNA. Unreplicated nucleosomal DNA reads display an even distribution along the chromosome while nascent nucleosomal DNA shows specific enrichment to previously annotated origins of replication. **C)** Replication profiles from previously annotated origins of replication for chromosome 13 identified by S-phase copy number (Yabuki et al. 2002). **D)** Total number of nascent (HL) nucleosomal dyads per bp along chromosome 13 for a late time point, 90min post release from G₁ into S-phase. Reads show a more even distribution along the chromosome indicating that S-phase has completed. **E)** Distribution of nascent (HL), smoothed reads (10,000bp moving average) per base pair along chromosome 13 for a late 90min time point post release into S-phase.

3.2.7 Nascent chromatin isolation by thymidine analogue substitution

Complimentary to stable isotope labelling, isolation of nascent nucleosomal DNA was approached using thymidine analogues. While CsCl gradient ultracentrifugation of isotopically labelled DNA has been demonstrated to isolate replicated nucleosomal DNA, the approach is a time consuming and cumbersome technique. The resolving power of CsCl gradients diminishes with fragment size, possibly allowing cross-contamination of isolated nascent and unreplicated nucleosomal DNA. Additionally, analysis of nascent chromatin organisation by two different methods would give greater significance to common observations between both techniques.

To this end isolation of nascent chromatin was approached by incorporation of the thymidine analogue 5-ethynyl-2'-deoxy-uridine (EdU). This analogue differs from thymidine only at the 5' position of the nucleobase where a terminal alkyne group replaces the methyl group. Importantly, DNA polymerase will incorporate exogenous EdU in place of thymidine on the nascent DNA strand (Salic and Mitchison 2008). While fungi do not have a thymidine salvage pathway, incorporation of exogenous EdU into DNA is facilitated by use of a yeast strain containing the human equilibrative nucleoside transporter 1 (hENT1) along with herpes simplex virus thymidine kinase (tK). Thymidine kinase (tK) is required to phosphorylate thymidine to dTMP where it enters the cellular nucleotide pool and is incorporated into DNA. The thymidine transporter allows efficient entry of thymidine into the cell (Lengronne, Pasero et al. 2001, Sivakumar, Porter-Goff et al. 2004). Subsequently purified EdU labelled DNA is made amenable for isolation by covalent attachment of a biotin-azide through a Cu(I)-catalyzed [3+2] cycloaddition reaction ("click" chemistry) which serves as an epitope for isolation by streptavidin pull-down (Rostovtsev, Green et al. 2002). Previously this approach has been applied to visualising DNA replication following

cycloaddition to fluorescent-azides rather than biotinylated azide (Salic and Mitchison 2008).

To apply this approach to isolate nascent nucleosomal DNA a yeast strain competent in metabolising EdU was arrested in G₁ using α -factor followed by release into an EdU containing medium and harvesting cells at defined S-phase time points. Nucleosomal DNA isolated by MNase digestion was then biotinylated using biotin-azide in the presence of copper using the “click chemistry” reaction (figure 3.8). Finally the biotinylated nascent DNA was pulled down using magnetic streptavidin and processed for Illumina high throughput sequencing.

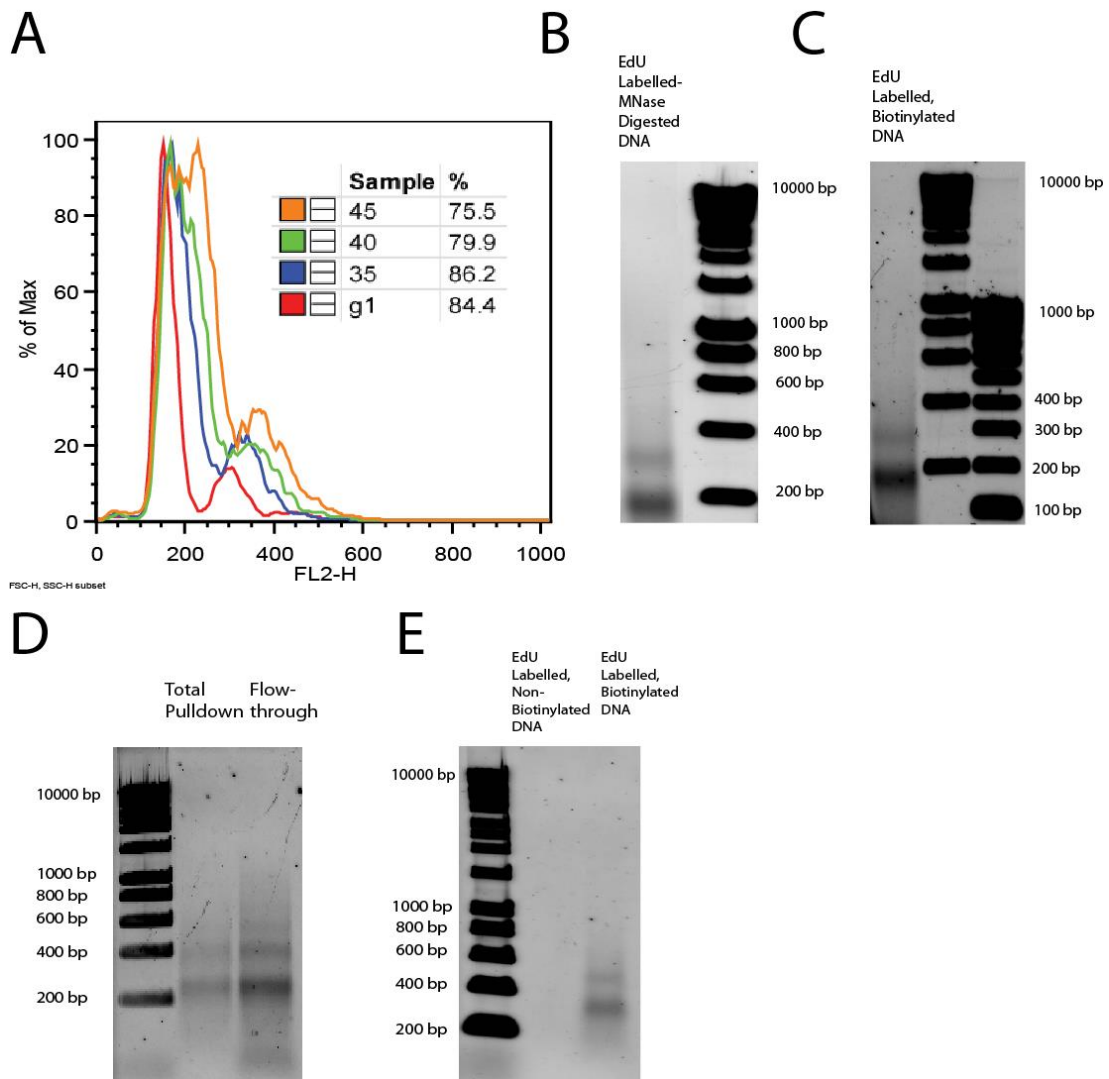


Figure 3-8 - Isolation of nascent chromatin via pull-down of EdU labelled DNA. **A)** FACS analysis of a yeast culture harvested at defined time points following release from G₁ arrest in an EdU containing S-phase medium. It can be seen that the fluorescently labelled DNA content of the yeast culture increases over S-phase as the DNA fluorescence peak migrates from 1C (FL2-H 200) to 2C (FL2-H 400). **B)** Cells were grown for approximately one replicative cycle, ~60min post release from α -factor arrest in a 50 μ M EdU containing medium. Formaldehyde fixed cells were subject to 50U of MNase treatment resulting in a ladder of predominantly mono (~150bp) and di (~320bp) nucleosomal DNA fragments. **C)** 10 μ g of nucleosomal DNA was conjugated to biotin-azide in a 3+2 cyclo-addition reaction. **D)** Pull-down of biotin conjugated nucleosomal DNA. Streptavidin bound material is shown in lane 2 (total pull-down) while DNA which did not bind the streptavidin is present in lane 3 (flow through) **E)** Pull-down of 10 μ g of EdU labelled DNA (from A) which is conjugated to biotin (lane 3, biotinylated DNA) and not conjugated to biotin (lane 2, Non-biotinylated DNA). Only biotin conjugated DNA is specifically pulled down by the magnetic streptavidin beads.

Application of these approaches, as seen in figure 3.8 does indeed facilitate isolation of EdU labelled nascent nucleosomal DNA. Flow cytometry analysis of a synchronised S-phase culture in EdU containing medium shows that replication proceeds unperturbed in the presence of the thymidine analogue. The specificity of the streptavidin pull-down is shown by the observation that only biotin conjugated nucleosomal DNA is isolated by the magnetic streptavidin. This shows that streptavidin does not spuriously bind non-replicated DNA as native thymidine cannot be biotinylated by the click reaction. Hence it was decided to collect nascent nucleosomal DNA from synchronised cell cultures at defined S-phase time points and assess their nucleosome positioning genome-wide using paired end Illumina sequencing. The input material for each pull-down, referred to as total chromatin was also collected to act as a comparison for the nascent nucleosomal DNA isolated.

3.2.8 Validation of EdU based isolation of nascent chromatin using genomics

As described for CsCl based isolation of nascent nucleosomal DNA, subsequent to high throughput sequencing of non-replicated (total) and nascent nucleosomal DNA it was decided to test the data to ensure that the DNA isolated by EdU incorporation was indeed replication specific. To this end the number nucleosomal dyads (fragment read midpoint) was plotted as a function of base pair along yeast chromosome 13 (figure 3.9). At time points early in replication nascent reads are enriched at specific chromosome locations that correlate with previously defined origins of replication (figure 3.9B,3.9D). Early nascent read enrichment for the same chromosome compares favourably to sort-seq data (Muller, Hawkins et al. 2014) with a Pearson correlation 0.732 increasing to 0.906 for later S-phase time points.

In contrast, the total chromatin reads for the same time point display an even distribution of reads across chromosome 13 (figure 4.9C) further suggesting that isolated nucleosomal DNA by EdU incorporation is replication specific.

In addition, nascent read enrichment from EdU and CsCl based approaches also correlate highly with each other (Pearson correlation 0.820). This suggests indicating that these two differing methods indeed isolate replication specific chromatin by two different principles.

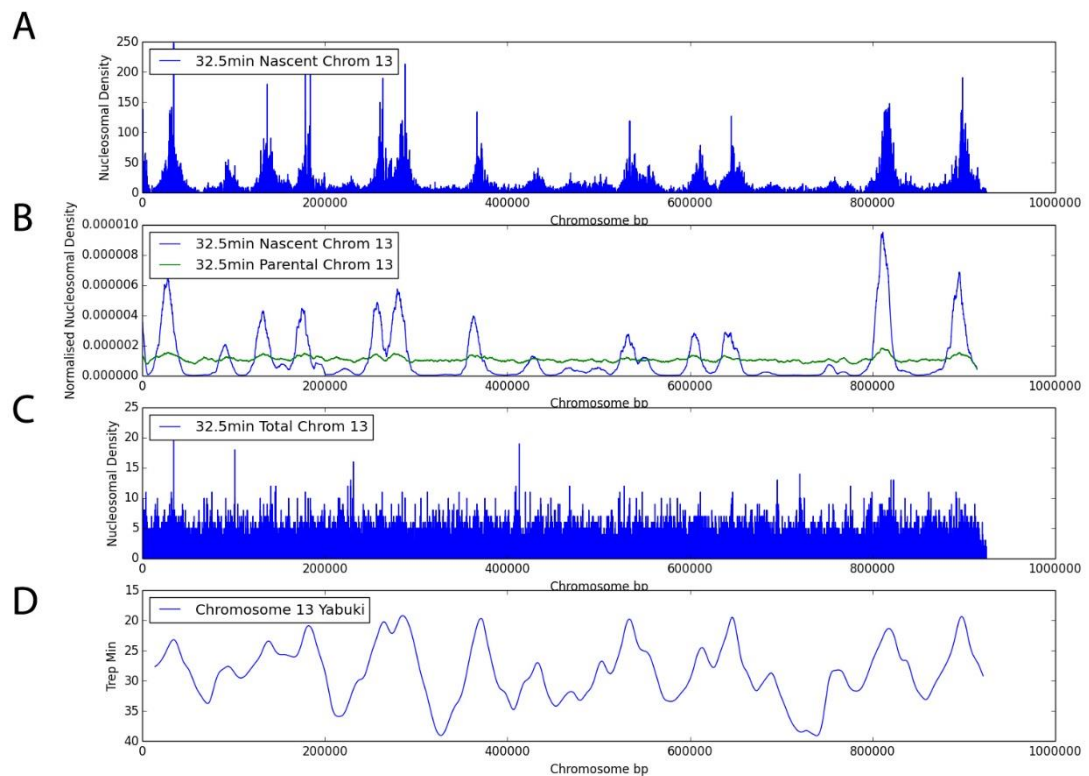


Figure 3-9 - Validation of nascent chromatin isolation by EdU incorporation using genomics. A) Total number of nascent nucleosomal reads per bp along chromosome 13 for an early S-phase time point, 32.5min post release from G₁. Distribution of nascent read enrichment along the chromosome is similar to replication profiles from previously annotated replication origins (D). **B)** Nucleosomal reads plotted as a 10,000bp moving average for replicating (nascent) and non-replicated (total) DNA. Non-replicated nucleosomal DNA shows an even distribution along the chromosome while nascent nucleosomal DNA shows specific enrichment to previously annotated origins of replication. **C)** Total number of reads per bp along chromosome 13 for total chromatin (non-replicated) at an early S-phase time point. **D)** Replication profile of previously annotated origins of replication for chromosome 13 identified by S-phase copy number (Yabuki et al. 2002).

3.2.9 Comparison of EdU labelling and CsCl gradient ultracentrifugation as approaches for nascent chromatin isolation.

Given that two different approaches were established as means of isolating nascent nucleosomal DNA, there was a desire to assess if one method was favourable over the other. We considered the normalised distribution of nascent nucleosomal reads along chromosome 13 for two similarly early time points post release from G₁ arrest into S-phase (figure 3.10). Considering first the EdU pull-down approach, there is a large ratio in read depth between regions surrounding replication origins and regions that are passively replicated in late S-phase. This is not seen to the same extent using the CsCl gradient approach. Diminished enrichment proximal to origins and greater read depth in typically late replicating regions may indicate slight contamination of isolated nascent DNA with non-replicated material from the CsCl gradient. This is a likely scenario given the observed smearing of small DNA fragments across multiple fractions of CsCl gradients (figure 3.3).

The presence of non-replicated DNA may contribute to higher read depth across the late replicating regions where one would not expect nascent reads during early replication. Increased read depth across the chromosome could lead to diminished read enrichment around known origins of replication. These findings hint that the EdU pull down approach is the more sensitive of the two methods as it does not appear to isolate non-nascent DNA as readily as the CsCl based approach.

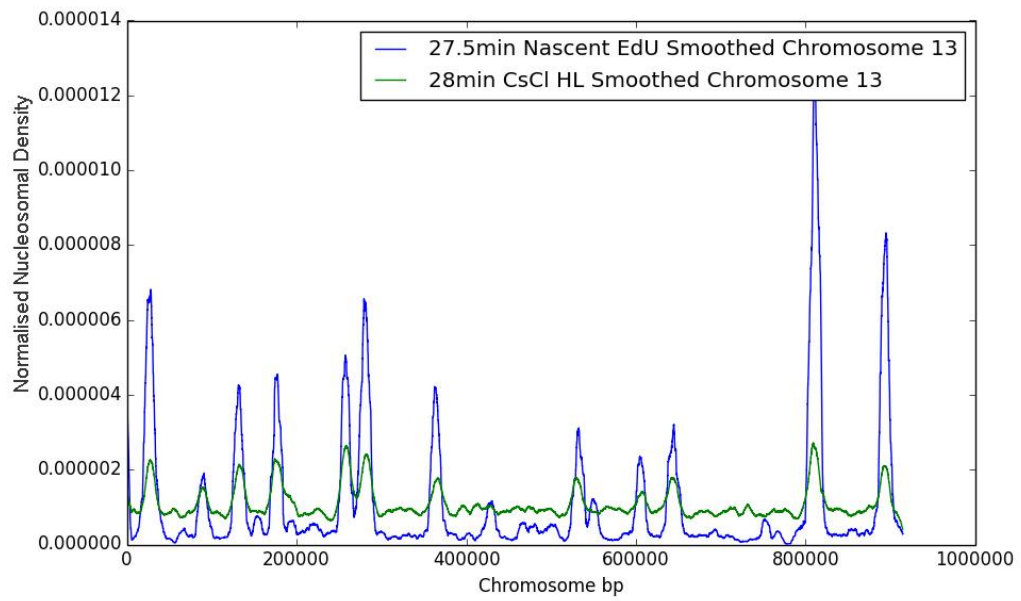


Figure 3-10 - Comparison of CsCl gradient ultracentrifugation and EdU labelling as approaches for nascent nucleosomal DNA isolation. Normalised smoothed (10,000bp moving average) reads for early S-phase time points were plotted as a function of chromosomal co-ordinate (bp) along chromosome 13. In comparison to the EdU based approach for a similarly early time point, the CsCl based approach shows a much lower read enrichment around replication origins compared to the read depth along typically late replicating regions of the chromosome.

3.3 Discussion

Deciphering primary level chromatin architecture has been made viable with the onset of deep sequencing technologies (Zhang and Pugh 2011). DNA fragments may be isolated which are representative of histone octamer contacts post treatment with the endo-exonuclease, MNase (Hewish and Burgoyne 1973). High read depth sequencing allows one to accurately map the profile of nucleosome organisation genome-wide (Yuan, Liu et al. 2005, Lee, Tillo et al. 2007). Transfer of these established methods to replicating chromatin requires sequencing of nascent nucleosomal DNA fragments. This has resulted in the adaption of two techniques previously used to monitor DNA replication. First is the classical Meselson-Stahl approach, involving differential density labelling of DNA using stable isotopes coupled with CsCl gradient ultracentrifugation (Meselson and Stahl 1958). Secondly, labelling of the nascent strand using a thymidine analogue (Salic and Mitchison 2008) was employed which facilitates a replication specific pull-down of nascent DNA.

Initial trial separations using HH and LL labelled DNA by CsCl gradient ultracentrifugation (figure 3.2) revealed that it was possible to attain adequate separation of DNA fragments across a range of molecular weights as described in the literature (McCarroll and Fangman 1988). When applying this approach to HH and HL labelled nucleosomal DNA it is apparent that separation efficiency is reduced (figure 3.3). There is an expected loss in separation potential based upon the reduced mass difference between HH and HL labelled DNA in comparison to HH and LL. However, the inefficient separation observed was most notable for shorter DNA fragments which were present across multiple fractions of the CsCl gradient (figure 3.3).

There are two likely scenarios causing such an effect. Short DNA fragments may resist being brought to equilibrium as they may more readily diffuse through the Cs⁺ ions which form the gradient (Birnie and Rickwood 1978). This smearing effect is likely to occur when the force of centrifugation is removed and the ultracentrifugation rotor is brought to rest. Secondly, the GC% of smaller fragments deviate further from the genome-wide average. This could result in density based separation of GC and AT rich sequences for both HH and HL labelled species resulting in a smear across the gradient for mono-nucleosome sized DNA fragments (Figure 3.3 D) (Birnie and Rickwood 1978).

Most published methods describing centrifugal separation of isotope labelled fragments involve the use of an initial high speed spin to generate an equilibrium gradient. This ensures labelled DNA is brought to buoyant equilibrium with Cs⁺ ions in the gradient. This is followed by a lower speed spin ending with a gentle deceleration to rest in the absence of an applied brake. During the deceleration phase there is an opportunity for the CsCl gradient to relax to form a shallower gradient (of Cs⁺ ions) capable of resolving DNA fragments with smaller differences in density. Work by Anet and Strayer has shown that the beneficial effect of gradient relaxation is coupled with increased diffusion of small fragments (in comparison to large DNA fragments) through the gradient during slow deceleration (Anet and Strayer 1969). As a result it was decided to perform subsequent centrifugations with a more severe braking procedure to help smaller DNA fragments to maintain appropriate equilibrium within the gradient.

In order to increase the resolution of nascent and parental DNA separation, heavy labelling of parental DNA was improved in order to increase the mass difference between HH and HL labelled DNA. Increased mass difference between labelled DNA

species should result in increased partitioning within the gradient and potentially compensate for the intrinsic problems of CsCl ultracentrifugation as outlined above.

Increased heavy labelling was achieved in two ways. Firstly, the glucose isoform D-glucose- $^{13}\text{C}_6,1,2,3,4,5,6,6\text{-d}_7$ was used as a carbon source in place of ^{13}C containing glucose. This isoform allows carbon and hydrogen labelling when metabolised into DNA. The isoform allows hydrogen labelling at non-exchangeable sites with deuterium. The extra deuterium incorporation should increase the mass difference between HH and HL DNA from 13 to 18Da (Sivakumar, Porter-Goff et al. 2004).

This new isoform was used in a yeast strain capable of producing the nucleobases adenine and uracil. Their biosynthesis leads to more efficient isotope labelling of DNA as it is not composed of exogenously (isotopically light) added nucleobases to the heavy labelling media (figure 3.4-3.5). Indeed, DNA quantification of gradient fractions from strains capable of uracil (figure 3.5A) or both uracil and adenine (figure 3.5B) biosynthesis indicates that the separation of nascent and non-replicated fragments is increased. Increased refractive index values for collected peak HH fractions indicates increased mass labelling of parental DNA has occurred. This allows the increased mass differences between HH and HL to be exposed by the ultracentrifugation process.

Complementary to the use of isotope labelling and CsCl gradients, nascent DNA was also isolated by use of the thymidine analogue 5-ethynyl-2'-deoxy-uridine (EdU). Using a competent strain, EdU is incorporated in place of thymidine on the daughter strand. Isolated nascent nucleosomal DNA is covalently coupled to a biotinylated azide which allows isolation of replication specific material via streptavidin pull down *in vitro* (figure 3.8). EdU was chosen as an analogue to label nascent DNA due to its

similarly to canonical thymidine. Other thymidine analogues containing viable epitopes such as BrdU (5-bromo-2'-deoxyuridine) were not considered as their large halogenated groups have been shown to influence nucleosome positioning *in vitro* (Miki, Shimizu et al. 2008, Miki, Shimizu et al. 2010). EdU properties differ from standard deoxy-thymidine minimally while the biotin marker is attached to DNA post chromatin digestion with MNase *in vitro*. This is of particular importance given the proposed role of DNA sequence dependent nucleosome positioning (Segal, Fondudé-Mittendorf et al. 2006, Zhang, Moqtaderi et al. 2009).

Post Illumina sequencing of non-replicated and nascent chromatin from S-phase time points, the replication specificity of both methods can be tested using genomics (figure 3.7,3.9). At early S-phase both approaches demonstrate nascent read enrichment profiles comparable to previously described replication profiles (Yabuki, Terashima et al. 2002, Muller, Hawkins et al. 2014). As expected non-replicating chromatin shows no such enrichment.

In comparing both methods (figure 3.10) it would appear that the EdU approach is more favourable. Given similarly early S-phase time points the EdU based approach has greater read enrichment in the vicinity of known replication origins in comparison to the CsCl approach which shows greater read depth for typically late replicating chromosomal regions. This result may be caused by small contaminating amounts of non-replicated nucleosomal DNA. If very little nascent DNA is present on the replicating side of the CsCl gradient during early replication, trace amounts of contaminating non-replicated (HH) material could be over represented during sequencing. A biased profile of nascent reads may explain reduced enrichment for origins of replication and increased reads depth along passively replicated regions of the genome. These observations have led us to favour the EdU labelling approach in

our characterisation of nascent chromatin. It is worth noting that data generated by the CsCl gradient approach could be improved by identifying HH regions of the genome that are likely to contaminate the HL region of a gradient during centrifugation. These data could be used to generate a baseline for identifying significant nascent nucleosomal reads (Alvino, Collingwood et al. 2007).

In conclusion, two different methods for monitoring DNA replication have been adapted to monitor primary level chromatin structure during DNA replication. The ability to assay nucleosome positioning using two different approaches is advantageous as reinforcing observations gives confidence in experimental findings. The results of which are explored in chapter 4.

4 Assessment of nascent chromatin structure during synchronised DNA replication

4.1 Introduction

Over the past decade the advent of genomic technologies has led to an explosion in the numbers of studies mapping nucleosome positions genome-wide. Pioneering studies using high resolution micro-arrays showed that nucleosomes have a distinct organisation and maintain specific arrangements (Yuan, Liu et al. 2005, Lee, Tillo et al. 2007, Schones, Cui et al. 2008). Nucleosome maps have been produced for over 30 different species and multiple cell types in multicellular organisms (Hughes and Rando 2014). Of these, the budding yeast, *Saccharomyces cerevesiae* is the best studied and the focus of attention in this study. Analysis of nucleosome density profiles has shown a specific organisation with respect to genes comprising of several features which are conserved from yeast to human (Yuan, Liu et al. 2005, Schones, Cui et al. 2008).

A popular model to explain the positioning of coding region nucleosomes involves genomic elements such the NDR acting as barriers against which adjacent nucleosomes are positioned. Initially it was suggested that nucleosomes were positioned statistically, whereby nucleosomes form ordered arrays against these barriers due to constraints from adjacent nucleosomes. The statistical positioning model also infers spacing of nucleosomes is inversely related to nucleosome density along the DNA template (Kornberg and Stryer 1988). Analysis of wild type *in vivo* datasets initially supported this model (Mavrich, Ioshikhes et al. 2008), however analysis of nucleosome organisation from reconstituted chromatin does not (Zhang, Wippo et al. 2011).

A key feature of models for positioning by exclusion is that the average spacing between adjacent nucleosomes is predicted to be dependent on nucleosome density. However, reconstituted chromatin using low levels of histones showed that canonical nucleosome spacing was not lost and that nucleosome density was retained at the beginning of genes (Zhang, Wippo et al. 2011). This led to a modified statistical positioning model, referred to as the barrier packing mechanism being adopted. This model suggests that nucleosomes are actively stacked by trans-acting factors against a genomic barrier element, but not in locations that result in packaging closer than around 15 base pairs (Zhang, Wippo et al. 2011).

Nislow and colleagues interrogated the issue through analysis of genic nucleosomes during a histone H4 depletion time course. These experiments showed that TSS proximal nucleosomes display shifts in position, notably shifting of the +1 away from the TSS (van Bakel, Tsui et al. 2013). By carefully analysing the positioning of arrays of nucleosomes at single loci van Bakel et al. noticed that defects in the organisation of nucleosomal arrays were not propagated to adjacent nucleosomes suggesting that nucleosomes can be positioned independently of each other (van Bakel, Tsui et al. 2013). These observations indicate that further refinement of the barrier packing mechanism is required.

The fact that nucleosome organisation is centred on promoters provides a strong indication that the organisation of nucleosomes over coding regions is in some way coupled to transcription. Consistent with this there are links between Chd1, ISW1a and transcription elongation (Simic, Lindstrom et al. 2003, Smolle, Venkatesh et al. 2012). Histone chaperones such the FACT complex have been shown to co-localise with elongating RNA polymerase II (Mayer, Lidschreiber et al. 2010) and defects to chromatin structure in the absence of FACT are magnified in highly transcribed genes

(Jamai, Puglisi et al. 2009). Furthermore, removal of RNA polymerase II in yeast results in loss of genic nucleosome positioning distal to the promoter (Weiner, Hughes et al. 2010). Transcription rates correlate with NDR width at highly transcribed genes (Shivaswamy, Bhinge et al. 2008) while lowly transcribed genes in budding yeast display a moderate genic nucleosome depletion (Radman-Livaja, Ruben et al. 2011). In addition, shifting of nucleosomes distal to the promoter is exacerbated at highly transcribed genes when RNA polymerase II is removed (Weiner, Hughes et al. 2010).

If chromatin is organised in a reaction coupled to transcription one question that arises is; how are nucleosomes arranged following DNA replication before they are first transcribed? The extent to which chromatin organisation is inherited from one generation to the next is of key importance when it is considered as a vehicle for non-genetic inheritance. Potentially, the ability to observe chromatin between conversion from one state to another could also provide new insights into the coupling between chromatin organisation and transcription.

To this end we have isolated nascent nucleosomal DNA over the course of S-phase in synchronised cultures using two different approaches (outlined in chapter 3) and attempted to annotate the nature of this nascent chromatin organisation.

4.2 Results

4.2.1 Assessment of nascent chromatin structure aligned to coding regions

Having established systems with which we could isolate recently replicated nucleosome length DNA fragments, we next applied this to study the organisation of nucleosomes on nascent DNA. As nucleosomes show strongest organisation flanking promoters (Yuan, Liu et al. 2005, Lee, Tillo et al. 2007), nucleosomal dyad frequencies were first plotted for yeast genes aligned via their transcription start sites.

This was initially performed for non-replicated (HH) and nascent (HL) DNA at 33, 41, 60 and 90 min time points post release from G₁ arrest isolated using the CsCl ultracentrifugation approach (figure 4.1). Comparison of non-replicated and nascent chromatin shows only subtle differences between the two states. At early time points (33min) there is a reduction in occupancy (nucleosomal density/read depth) of the phased genic nucleosomes, an effect which is most pronounced for nucleosomes downstream rather than upstream of the TSS. Towards the end of replication (60min), phased coding region nucleosomes become more similar to non-replicated chromatin. Interestingly at later time points (60min, 90 min) the read depth of the nascent +1 nucleosome becomes greater than the non-replicated chromatin +1. While this result is reproducible between repeat experiments the observation is difficult to evaluate. At such a late time point post G₁ release the remaining non-replicated chromatin may arise from inviable cells which did not enter DNA replication post α -factor release.

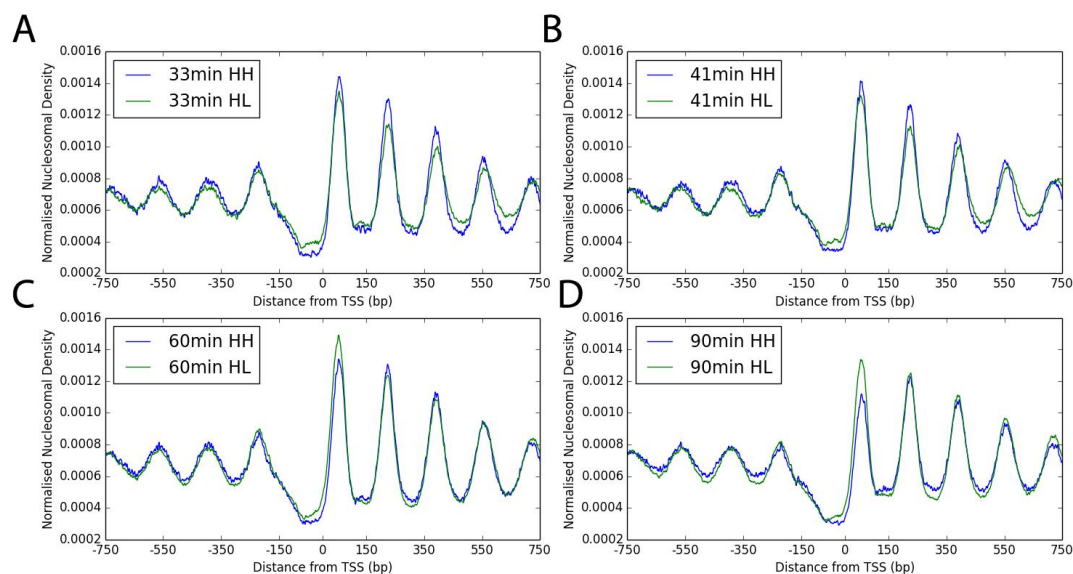


Figure 4-1 - Nucleosome positioning on replicated and unreplicated DNA isolated by CsCl gradient ultracentrifugation. The normalised frequency of nucleosome dyads aligned to the TSS of all genes at **A)** 33min, **B)** 41 min, **C)** 60 min and **D)** 90 min following release from G₁ arrest. Replicated (HL) and non-replicated (HH) DNA is shown for each time point.

A similar scenario occurs when analysis is performed on chromatin which has been isolated via pull down of EdU labelled nascent DNA (figure 4.2). Again early nascent genic nucleosomes (35min) display lower read depth and an increase in nucleosome density for the putative linker DNA. S-phase progression is again characterised by increased occupancy for coding region nucleosomes with removal of nucleosomal density from the linker regions (45min).

While both approaches indicate a subtle maturation during the course of replication, the presence of a significant nucleosome organisation at the earliest time points suggest there is a very rapid pathway for chromatin reassembly and nucleosome positioning. At all time points the nucleosome depleted region at the TSS is flanked by a dominant +1 nucleosome and an array of nucleosomes that are progressively less well positioned further into the coding region.

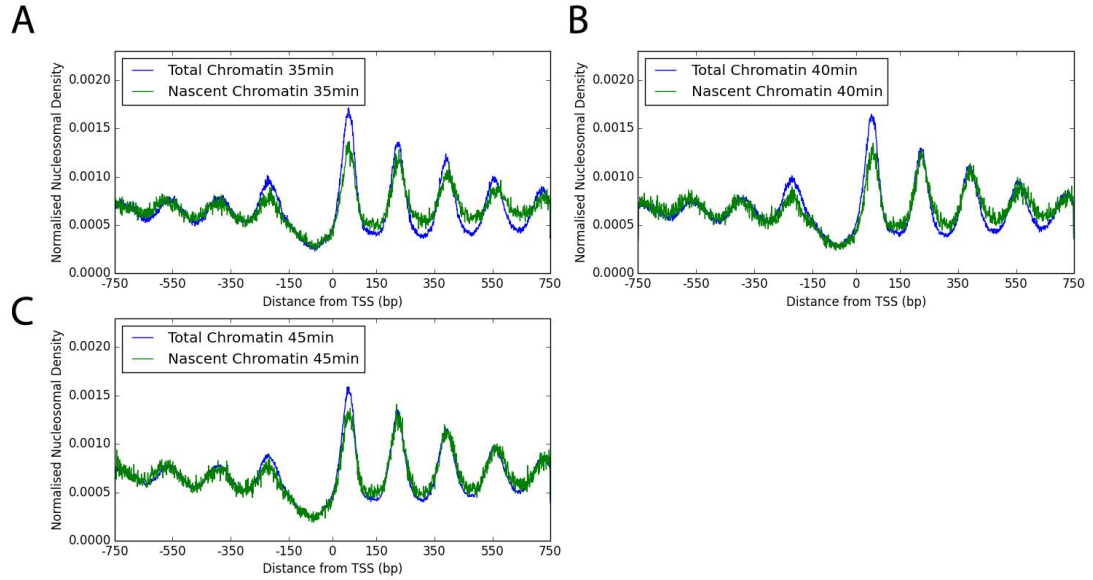


Figure 4-2 - Nucleosome positioning on replicated and non-replicated DNA isolated by EdU incorporation. The normalised frequency of nucleosome dyads aligned to the TSS of all genes at **A)** 35min, **B)** 40 min and **C)** 45min following release from G₁ arrest. Replicated (nascent) and non-replicated (total) DNA is shown for each time point.

4.2.2 Influence of transcription on nascent chromatin maturation

To investigate the coupling of the process of chromatin maturation to transcription, TSS aligned nucleosome profiles were generated for the top and bottom 10% of genes ranked by expression levels on nascent chromatin (figure 4.3 and 4.4).

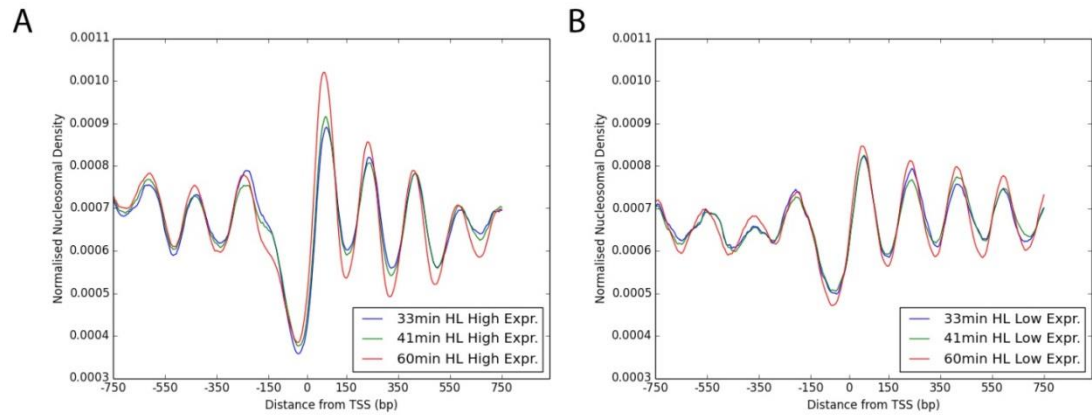


Figure 4-3 - Chromatin organisation during DNA replication at genes transcribed at high and low levels assessed by CsCl gradient ultracentrifugation. Nucleosome density plots of nascent chromatin aligned to the TSS of the top 10% (High Expr.) and bottom 10% (Low Expr.) of genes ranked by gene expression. **A)** Normalised frequency of replicated (nascent) nucleosome dyads aligned to the TSS for highly expressing genes at 33, 41 and 60min S-phase time points. **B)** Normalised frequency of replicated (nascent) nucleosome dyads aligned to the TSS for lowly expressing genes at 33, 41 and 60min S-phase time points.

Using both the CsCl and EdU approaches, the earliest S-phase time points at both highly and lowly transcribed genes are assembled within phased nucleosomal arrays (figures 4.3A,B and 4.4A,B). Throughout S-phase, highly transcribed genes are characterised by a more prominent NDR. The nucleosomal oscillation is also more pronounced especially at the +1 and +2 nucleosomes. These features become more prominent at later time points, when there will have been a greater opportunity for these genes to have been transcribed.

In support of this result, hundreds of genes that change expression during T cell stimulation demonstrated that the major change in chromatin structure at the higher expression level was an increase in occupancy of the +1 nucleosome (Schones, Cui et al. 2008).

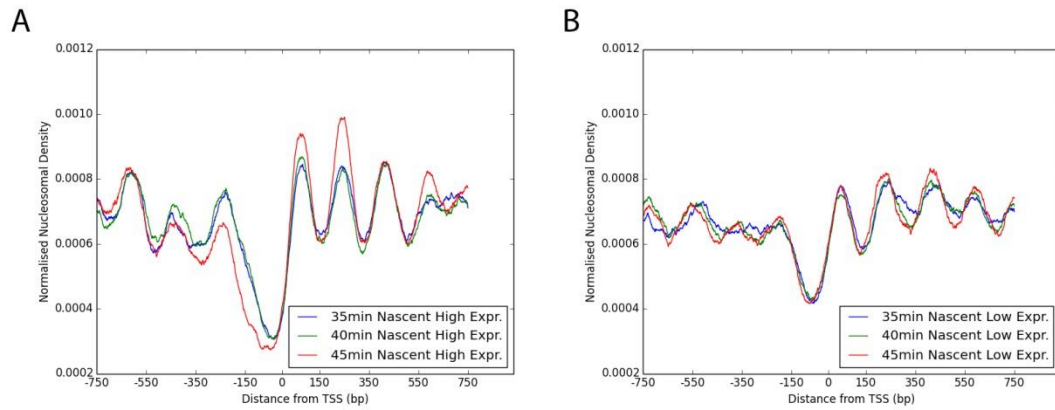


Figure 4-4 - Chromatin organisation during DNA replication at genes transcribed at high and low levels assessed by EdU incorporation. Nucleosome density plots of nascent chromatin aligned to the TSS of the top 10% (High Expr.) and bottom most 10% (Low Expr.) of genes ranked by expression level. **A)** Normalised frequency of replicated (nascent) nucleosome dyads aligned to the TSS of highly expressed genes at 35, 40 and 45min S-phase time points. **B)** The normalised frequency of replicated (nascent) nucleosome dyads aligned to the TSS of lowly transcribed genes at 35, 40 and 45min S-phase time points.

While high levels of transcription may develop directionality in nucleosome phasing during chromatin maturation, it does not describe adequately the initial assortment of positioned nucleosomes. This is evident when considering lowly transcribed genes in early S-phase (figure 4.3B and figure 4.4B) which are positioned but unlikely to have been transcribed so soon post replication. These observations suggest the existence of a post replication ground state that is organised with respect to transcribed genes before they have been transcribed.

The above analysis indicates that transcription levels have a minor “polishing” effect on chromatin maturation (figure 4.3 and figure 4.4). However, the data used to determine transcription activity was derived from asynchronous cultures (Lee, Tillo et al. 2007). As many genes exhibit transcriptional activity dependent on the cell cycle, we next considered expression at specific cell cycle stages (Rowicka, Kudlicki et al. 2007). Considering nascent chromatin is assembled in S-phase, genes which are specifically expressed outside of S-phase are not expected to be transcribed on the nascent template.

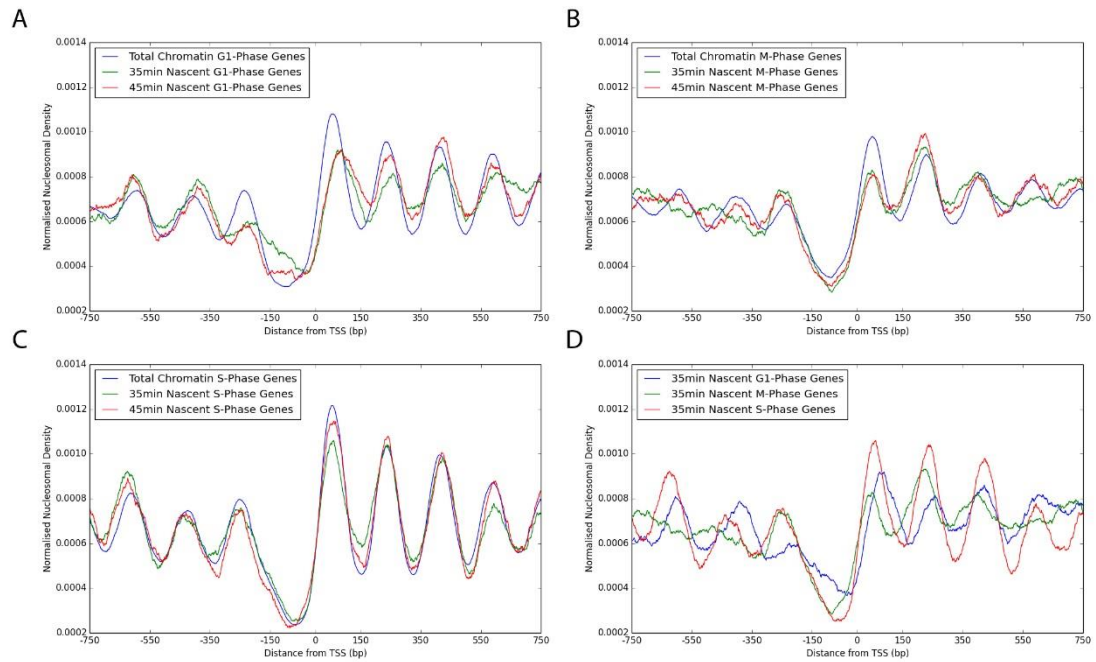


Figure 4-5 - Effect of transcription activation and repression on nascent chromatin isolated by EdU incorporation. The normalised frequency of non-replicated (total) and replicated (nascent) nucleosomal dyads aligned to the TSS of cell cycle regulated genes which are specifically expressed in **A)** G₁ phase **B)** M phase and **C)** S-phase at specific time points following release of a culture from G₁ arrest into S-phase. **D)** Comparison of cell cycle specific genes at an early S-phase time point.

Loss of positioning and occupancy are apparent for nascent nucleosomes in genes which are not expressed (G₁ and M) (figure 4.5A-B). This is particularly noticeable for G₁ specific genes where the +1 nucleosome maxima is of phase with the non-replicated +1 maxima, indicating that a change in positioning can be detected. In addition increased nucleosomal density in the NDR is an indication of irregular promoter architecture. M-phase specific genes show a slightly more canonical organisation although positioning of the downstream +4 nucleosome appears lost in early S-phase (figure 4.5B). This is illustrated by a loss of the nucleosomal oscillation at this region. S-phase specific genes (figure 4.5C) demonstrate well positioned coding regions nucleosomes which undergo subtle gains in +1 nucleosome read depth during replication.

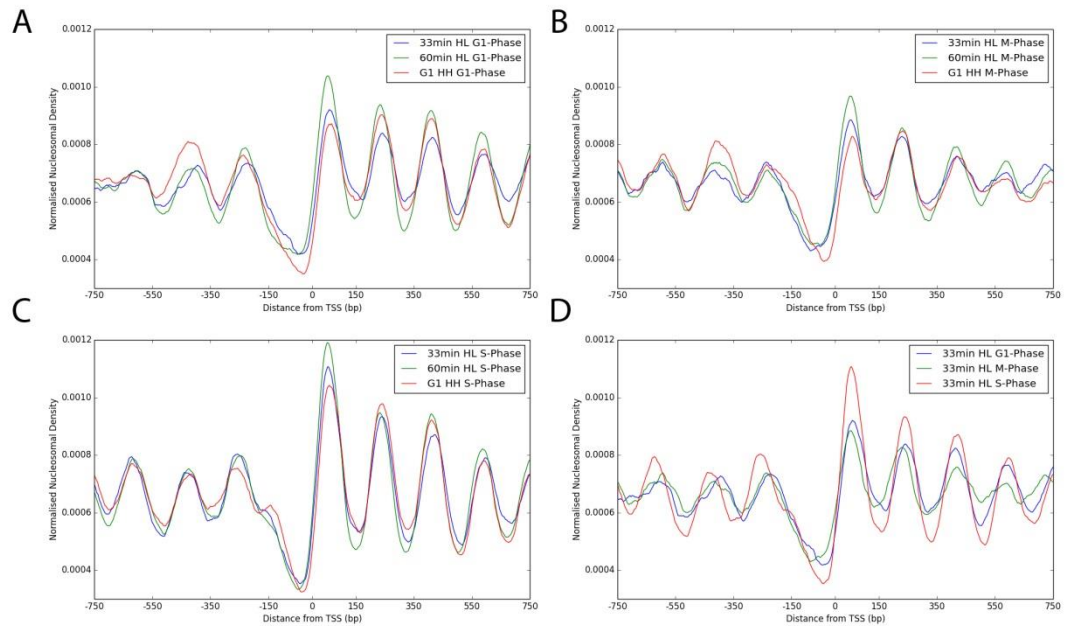


Figure 4-6 - Effect of transcription activation and repression on nascent chromatin isolated by CsCl gradient ultracentrifugation. The normalised frequency of non-replicated (HH) and nascent (HL) nucleosomal dyads aligned to the TSS of cell cycle regulated genes which are specifically expressed in **A)** G₁ phase **B)** M phase and **C)** S-phase at specific time points following release of a culture from G₁ arrest into S-phase. **D)** Comparison of cell cycle specific genes at an early S-phase time point.

Nascent chromatin isolated via CsCl centrifugation illustrates a similar result (figure 4.6). Nascent chromatin of genes transcribed in S-phase are well organised even at the start of S-phase and show little maturation (figure 4.6C). In contrast nascent chromatin in genes expressed outside of S-phase is less well organised especially at early S-phase time points (figure 4.6D) and undergo a greater maturation as S-phase proceeds. However it is worth noting that arrays of nucleosomes are, even if positioned slightly aberrantly, assembled on nascent chromatin regardless of transcriptional activity.

4.2.3 Clustering identifies pronounced chromatin maturation

In the analysis performed above, the chromatin organisation for large cohorts of genes is averaged. A risk in performing such analysis is that the process of averaging could obscure distinct behaviours at subsets of genes. To investigate this further the process of chromatin maturation at single genes was studied using hierarchical clustering (de Hoon, Imoto et al. 2004), a process which organises genes into groups with similar

nucleosome density profiles. This was performed for an early S-phase time point (35min) on chromatin isolated by EdU incorporation. The resultant gene organisation was then applied to late S-phase (45 min) and non-replicated (total) chromatin with the goal of identifying genes which had undergone pronounced maturation during this timeframe (figure 4.7).

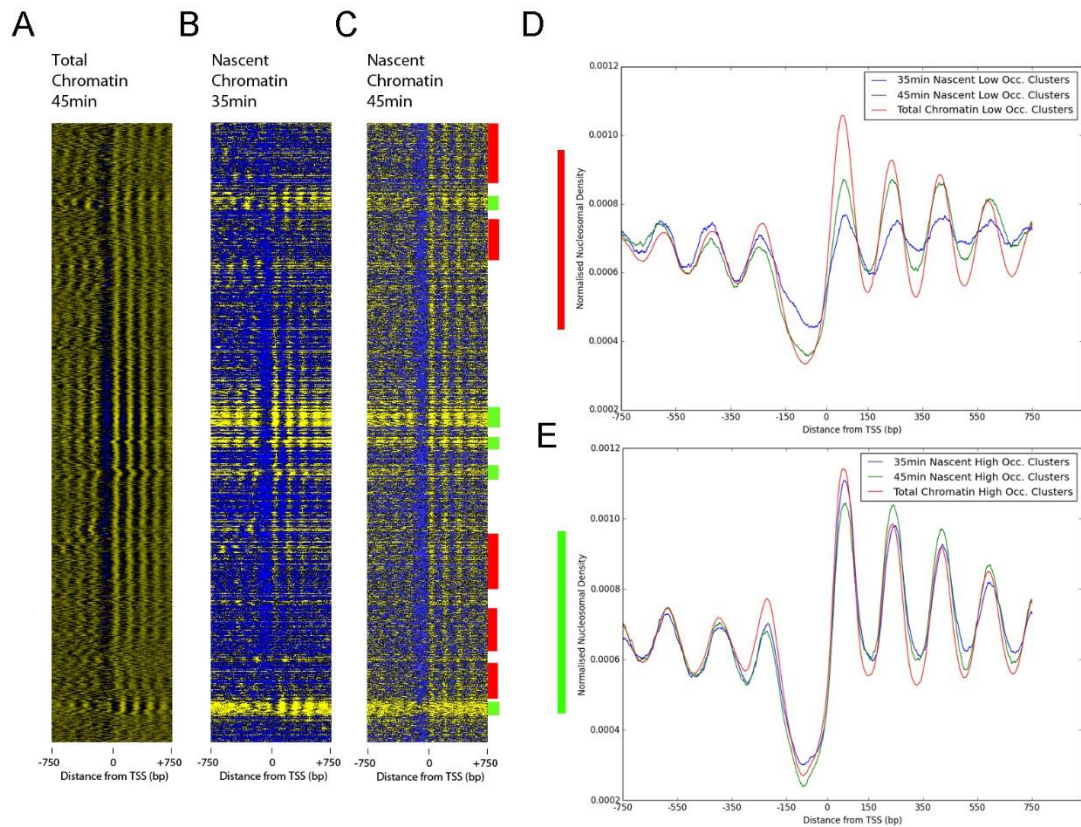


Figure 4-7 - Pronounced disruption of nucleosome organisation in genes identified by hierarchical clustering on replicating chromatin isolated via EdU incorporation. A-C) Heat maps of nucleosomal dyad frequencies (yellow) for all yeast genes sorted by order of genes attained from centroid clustering of the 35min nascent chromatin time point (B). Selected clusters of genes with high nucleosomal occupancy are marked by green bars (C). Selected clusters of genes with low nucleosomal occupancy are marked by red bars (C). **D)** Normalised frequency of nucleosome dyads aligned to the TSS of selected gene clusters (marked by red bar in C). **E)** Normalised frequency of nucleosome dyads aligned to the TSS of selected gene clusters (marked by green bar in C).

Comparison of heat maps in which genes from nascent chromatin are arranged in the same y-axis order (figure 4.7 B-C) allowed identification of ~1390 genes (marked red in figure 4.7C) with discernibly different nucleosomal arrangements along the coding region. Normalised nucleosome density profiles for these genes (figure 4.7D) describe severely disrupted chromatin for the early 35 min time point. This is illustrated by the heavily dampened nucleosomal oscillations, descriptive of a more random arrangement of genic nucleosomes. Furthermore a partially occupied NDR widens and becomes less nucleosome dense as replication proceeds, indicative of aberrant nucleosome removal. In addition gene clusters (marked green in figure 4.7) were also identified with canonical nucleosomal occupancy and spacing even at the earliest DNA replication time points (figure 4.7E).

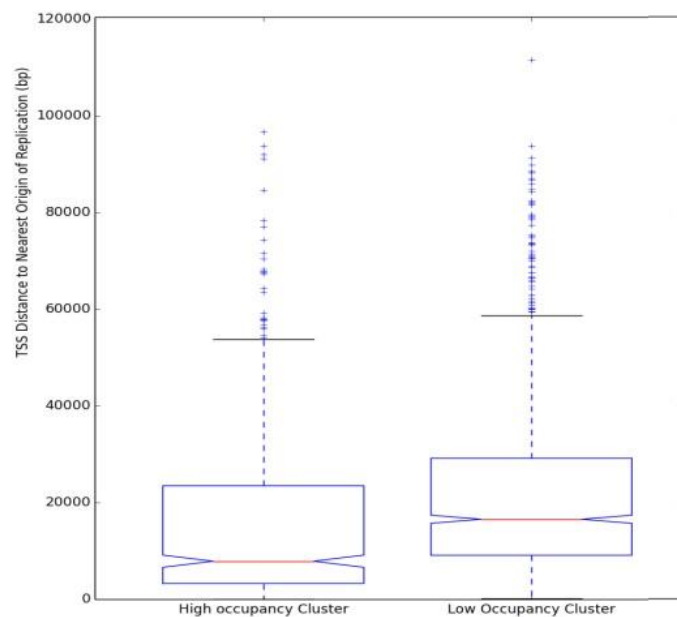


Figure 4-8 – Genes with poor nucleosome positioning during early S-phase are further from origins of replication. Boxplots displaying the distances from the TSS for high occupancy genes (figure 4.7C, green clusters) and low occupancy genes (figure 4.7C, red clusters) to the nearest origin of replication (Berbenetz, Nislow et al. 2010). Significance of the difference between boxplots is highlighted by non-overlapping notched medians. In addition the median distance of the high occupancy cluster does not overlap with the low occupancy cluster boxplot.

Analysis of genes with high (figure 4.7C, green clusters) and low nucleosomal occupancy (figure 4.7C, red clusters) showed no specific trends in terms of transcription activity or gene ontology (data not shown). However, the distance from the TSS to the nearest origin of replication (Berbenetz, Nislow et al. 2010) for genes with better chromatin organisation (higher occupancy cluster) at the early 35 min time point is less than those which have poor nucleosomal organisation at the same time point (figure 4.8).

This suggests that the selected genes of poor nucleosome organisation are located distal to origins of replication. The EdU labelled DNA isolated from these regions at earlier S-phase time points is likely to be most recently replicated. As a consequence, the nascent nucleosome organisation observed for these selected gene clusters may represent a transient state in which nascent chromatin is disrupted moments after assembly on the nascent template.

4.2.4 Clustering identifies +1 nucleosome depleted nascent chromatin

Characterisation of nascent nucleosome organisation has indicated that genic nucleosomes are phased in the same positions as observed in non-replicated “mature” chromatin. Current understanding regarding nucleosome organisation suggests that nucleosomes are actively packed against a fixed genomic barrier at the 5’ end of genes (Zhang, Wippo et al. 2011). Application of this model to chromatin assembly during DNA replication would see nascent nucleosomes packed against the NDR starting from the +1 position. Hence, one would expect +1 nucleosome organisation to be maintained or established first on nascent chromatin during S-phase.

To test the model in a replicative environment, genes were organised by hierarchical clustering of their +1 nucleosomes aligned at the highest occupancy dyad location. This was performed on +1 nucleosomes from nascent chromatin for the 35 min S-phase time point. The resultant y-axis ordering of genes obtained from centroid clustering of their +1 nucleosomes was applied to all considered datasets and visualised via heat map (figure 4.9A). Analysis of +1 nucleosome heat maps identified clusters of genes with abrogated +1 occupancy which were compiled into two groups (marked by purple and green bars, figure 4.9C). TSS aligned nucleosome density profiles were plotted for these two groups of genes (figure 4.9D-E)

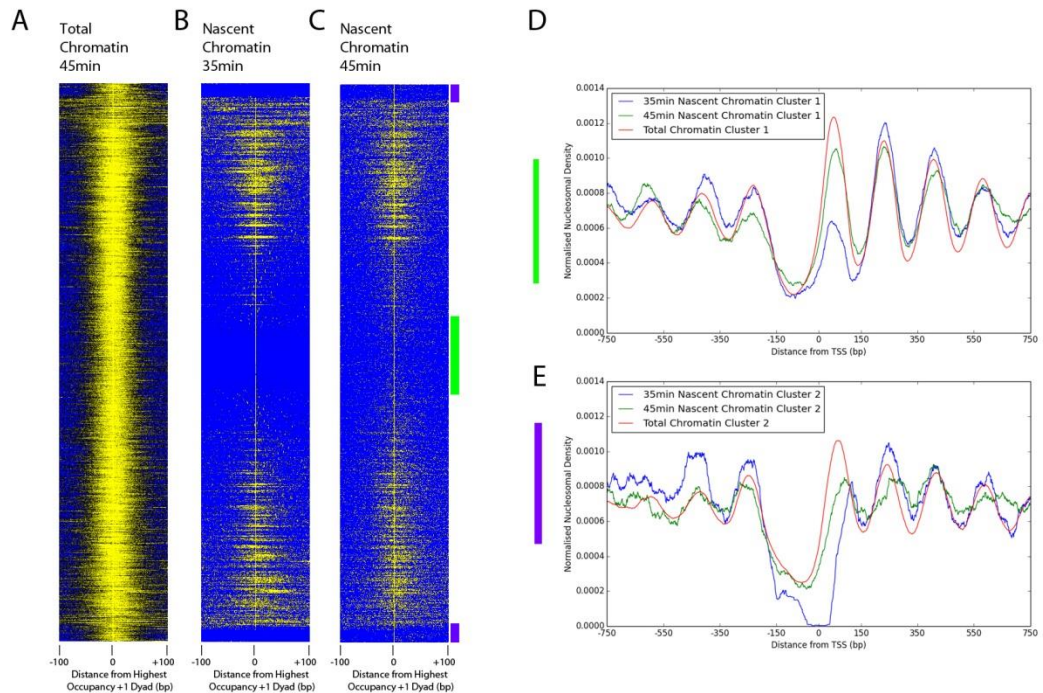


Figure 4-9 - Nucleosome positioning on genes identified by hierarchical clustering of +1 nucleosomes on nascent chromatin isolated by EdU incorporation. A-C) Heat maps of nucleosome dyad frequency for the +1 nucleosome of all genes. Y-axis order of genes was obtained by centroid clustering of +1 nucleosomes from the 35min nascent chromatin dataset (B) **D-E)** Normalised frequency of nucleosome dyads aligned to the TSS of selected gene clusters (marked by green and purple bars in C).

Nucleosome density plots for the first gene cluster (figure 4.9D) show that downstream coding region nucleosomes are well positioned, even at the earliest replication time point. Strikingly however, the nascent +1 nucleosome shows marked loss in occupancy at the earliest time point (35min) and gains occupancy during S-phase progression (45min). Diminished +1 nucleosome occupancy followed by ordered downstream nucleosomes suggests that nucleosomal packing does not initiate at the 5' end of genes as was previously considered (Kornberg and Stryer 1988, Zhang, Wippo et al. 2011).

The second gene cluster demonstrates a shift in +1 nucleosome positioning on nascent chromatin (figure 4.9E) during the 35min time point. This is apparent as the +1 nucleosomal maxima for the nascent and total chromatin samples are out of phase. As S-phase progresses the +1 nucleosome oscillation maxima progresses upstream toward the canonical position. This would imply that a correctly positioned +1 nucleosome is not compulsory for downstream genic nucleosome array formation. This provides further evidence that disruptions to nucleosome positions are not propagated to downstream nucleosomes. While the +1 nucleosome is not well positioned, downstream nucleosomes appear canonically phased on nascent chromatin at the early 35min S-phase time point (figure 4.9E). Similar findings have been observed previously whereby shifting of individual nucleosomes on genic arrays occurs without propagation of movement to adjacent nucleosomes (van Bakel, Tsui et al. 2013).

4.2.5 Nucleosome organisation surrounding origins of replication

In addition to transcription start sites, nucleosome organisation has been observed when aligned with respect to origins of replication (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010). The alignment of nucleosomal reads to ACS element of

replication origins illustrates a nucleosome organisation not dissimilar to that observed at coding regions. They are characterised by bi-directional arrays of nucleosomes that are arranged symmetrically with respect to the ACS (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010). Chromatin organisation surrounding replication origins has only been described in asynchronous and arrested cells. Hence it was decided to evaluate nascent nucleosome architecture at these sites during replication using the EdU labelling approach.

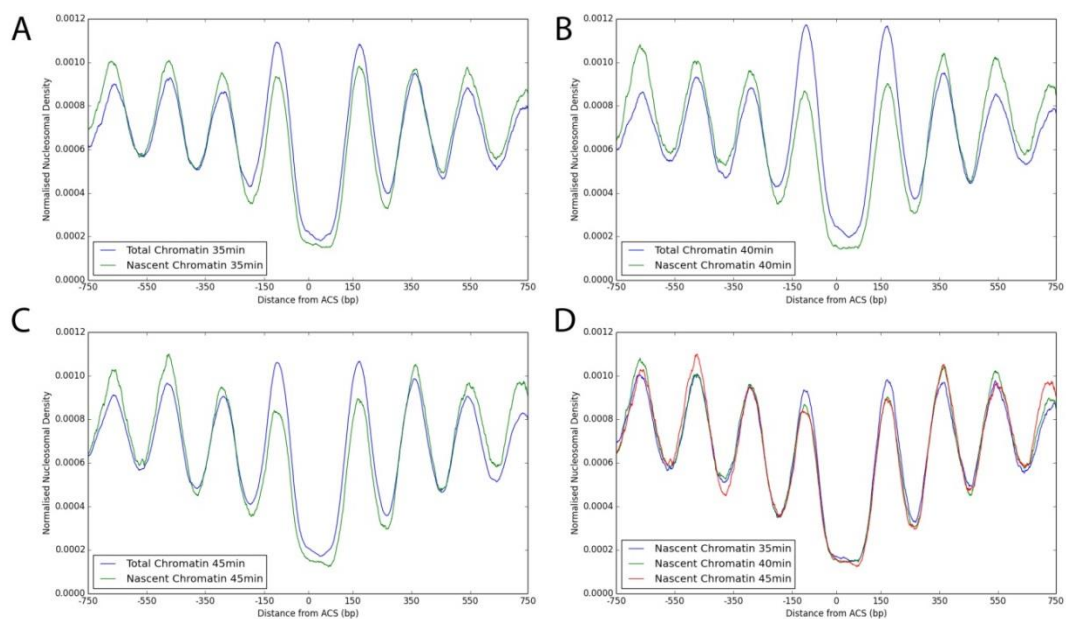


Figure 4-10 - Nucleosome positioning organised with respect to origins of replication in nascent chromatin isolated by EdU incorporation. Nucleosome density plots aligned to ACS elements 35min, 40 min and 45 min following release from G₁ arrest. **A-C)** Nucleosome density plots for non-replicated (total) and replicated (nascent) DNA at specified S-phase time points following release from G₁ arrest. **D)** Nucleosome density plot for replicated (nascent) DNA at specified S-phase time points following release from G₁ arrest.

Evaluation of nucleosome density profiles aligned to the ACS shows no obvious loss in organisation during replication. Nucleosomes from nascent and non-replicated chromatin are in well positioned, phased arrays extending up and downstream of the ACS. The -1 and +1 nucleosomes show decreased occupancy in comparison to non-replicated chromatin (figure 4.10A-C). However, nascent nucleosomal profiles describe no discernable changes over the sampled S-phase time points (figure 4.10D).

These results would suggest that nucleosome positioning surrounding replication origins is not drastically altered during DNA replication. This observation is likely due to the fact that isolated nascent DNA at replication origins has the longest opportunity to reorganise into mature chromatin following DNA replication.

4.3 Discussion

The past decade has seen significant advances in understanding nucleosome positioning genome-wide (Zhang and Pugh 2011). Nucleosomes appear best organised with respect to transcription and investigation of factors governing organisation with respect to the TSS has been the main focus of research as a means of describing nucleosome architecture genome-wide (Yuan, Liu et al. 2005, Lee, Tillo et al. 2007). It was initially suggested that nucleosomes were distributed statistically, evenly with respect to genetic boundary elements (Kornberg and Stryer 1988), however, modern approaches have allowed for a revised barrier packing mechanism in which nucleosomes are actively packed against the 5' ends of genes serving as a reference point to which downstream genic nucleosomes are arranged (Zhang, Wippo et al. 2011). However, *in vivo* histone depletion experiments revealed that packed nucleosomes at the 5' end of genes are not fixed and that nucleosomes can move without influencing the position of neighbouring nucleosomes on genic arrays (van Bakel, Tsui et al. 2013).

Assessment of nucleosome positions on nascent DNA allows the potential to observe if these models for nucleosome organisation apply during chromatin re-establishment. Furthermore it allows dissection of the relevant factors which have been shown to organise chromatin in asynchronous cultures. This was approached by assessing nascent chromatin isolated by CsCl gradient ultracentrifugation and pull-down of EdU labelled DNA as outlined in chapter 3.

Assessing all TSS aligned genes, only subtle changes in positioning are apparent between nascent and non-replicated (total) chromatin (figures 4.1 and 4.2). The observation that nucleosomes are organised at time points when DNA is anticipated to have been replicated for a short period of time suggests that the coupling between DNA replication, nucleosome assembly and repositioning is very rapid. Rapid repositioning of nucleosomes on nascent DNA has previously been observed on single loci using electron microscopy and nucleosome mapping by indirect end-labelling (Lucchini and Sogo 1994, Lucchini, Wellinger et al. 2001).

Nascent genic nucleosomes have slightly reduced density early in S-phase which recovers during replication progression. In contrast nucleosome density is lost from the putative linker regions as replication proceeds. Interestingly, the +1 nucleosome is most prominent in nascent chromatin, with progressively 3' nucleosomes gaining organisation as replication progresses. This could be regarded as being consistent with the proposed barrier packing model (Zhang, Wippo et al. 2011).

Transcription by RNA polymerase II (RNAPII) has been demonstrated to influence genic nucleosome positioning (Schones, Cui et al. 2008, Weiner, Hughes et al. 2010). Hence we considered the role of transcription as a process which may describe the modest changes seen when evaluating all genes (figure 4.3, figure 4.4). Both highly and lowly transcribed genes contain phased nucleosomal arrays for the earliest sampled S-phase time points. Replication progression is coupled with increased occupancy and organisation proximal to the 5' ends of highly transcribed genes, an event which is not observed on lowly transcribed genes. A similar result is observed when analysing cell cycle regulated genes (figure 4.5 and figure 4.6). Nascent chromatin for transcribing genes (S-phase) appears best organised during early

replication. However, phased nucleosomal arrays are also in place along the coding region of genes expressed predominantly in G₁ and M phase.

Evaluating genes based on transcription would suggest that initial nucleosome array formation is not transcription coupled. Forces which move to organise nucleosomes into canonical positions appear to act independent of a transcribing polymerase or associated factors. Nascent mRNA transcript mapping studies have suggested that moderately active genes are only transcribed ~7 times an hour (Pelechano, Chavez et al. 2010). Given that nascent chromatin is isolated minutes after S-phase initiation it is unlikely that any RNAPII activity will have occurred on lowly transcribed genes which already have ordered genic nucleosomal arrays.

Nevertheless the role of transcription activity on nascent chromatin is not to be understated. Maturation on actively transcribing chromatin appears to have a role in organisation of nucleosomes proximal to the 5' end of genes (figure 4.3 and 4.4). Moreover, initial rounds of transcription appear requisite for clearing of aberrantly positioned nucleosomes from the NDR (figure 4.5). This may potentially describe fine-tuning of the promoter region for efficient transcription pre-initiation complex assembly.

While nascent chromatin appears well organised when considering all genes, comparison of similarly organised genes (attained through hierarchical clustering) allows identification of distinct groups of genes which demonstrate a more drastic chromatin maturation (figure 4.7). These genes are characterised by diminished nucleosomal oscillations, indicative of a more random nucleosome organisation along the coding region. In addition these genes also contain a partially occupied NDR. Efforts to characterise these genes based on transcription activity or common gene

function proved fruitless. However when comparing well organised genes (figure 4.7E) and poorly organised, low occupancy gene clusters (figure 4.7D), it appears that genes with better nucleosome organisation are closer to origins of replication than those which are poorly organised during early S-phase (figure 4.8). Previously it has been observed that TSS proximal origins have earlier firing times during DNA replication (Berbenetz, Nislow et al. 2010). Considering an early S-phase time point, as genes proximal to origins are replicated first, they will have a longer timeframe to reorganise following replication fork passage than recently replicated genes that are distal to origins. In effect the isolated low occupancy cluster of genes may describe a more accurate representation of truly nascent nucleosome organisation post replication which is not seen when considering all genes.

An assumption of current models regarding genic nucleosome organisation is that the +1 nucleosome is a reference point with which downstream nucleosomes are organised (Mavrich, Ioshikhes et al. 2008, Zhang, Wippo et al. 2011). Reconstituted chromatin in the presence of diminished histone levels shows retention of positioning and occupancy of the +1 nucleosome (Zhang, Wippo et al. 2011).

Investigating assumptions of the barrier packing model on nascent chromatin further, +1 nucleosomes were organised by clustering. This allowed identification of two clusters of genes with obscured +1 organisation (figure 4.9). Analysis of these clusters (figure 4.9D) indicates that occupancy of the +1 is not retained during chromatin replication, nor is it vital for establishment of subsequent coding region nucleosome organisation. Furthermore, genes in the second gene cluster (figure 4.9E) have shifted +1 nucleosomes (35min) which do not appear to alter the phasing of downstream genic nucleosomes. Similar observations have been made by assessing genic nucleosome positions when chromatin remodelers have been removed and during histone H4

shutoff timecourse (van Bakel, Tsui et al. 2013). Taken together these results would suggest that the barrier packing model requires refinement or replacement in order to describe all features of nucleosome organisation that we and others have observed.

Finally, nucleosome organisation aligned to origins of replication was also evaluated as they represent another genetic element to which nucleosome organisation appears arranged with respect to (figure 4.10). Nascent chromatin surrounding these areas appears well maintained. Observation of well organised nascent chromatin proximal to origins is of little surprise considering this DNA is replicated first. Hence these regions have a longer timeframe to reorganise following assembly in a manner which based on previous observations is largely a transcription independent process.

Taken together, these results would suggest that nucleosome organisation is a rapid event, occurring potentially with passage of the replisome. While transcription has a belated role in the chromatin maturation process, it appears its effects are secondary to factors which phase nucleosomes on coding regions. While a distinct mechanism for chromatin maturation cannot be suggested, the findings of this report disagree with previous models of nucleosome packing. Such rapid assembly and maturation events make isolation and annotation of a truly nascent nucleosome organisation difficult. Efforts towards describing a truly nascent chromatin state are dealt with in chapter 5.

5 Assessment of nascent chromatin structure in asynchronous cultures

5.1 Introduction

The requirement of ATP to reconstitute *in vivo* nucleosome positioning using nuclear extracts intimated a role for ATP-dependent chromatin remodeling enzymes (Zhang, Wippo et al. 2011). Indeed studies using yeast strains carrying deletions for single and multiple chromatin remodelers showed dramatic loss of positioning for downstream genic nucleosomes (Gkikopoulos, Schofield et al. 2011).

A remodeler whose role in chromatin reorganisation during DNA replication that may prove insightful is the enzyme Function Unknown Now 30 (Fun30). Fun30 has been shown to act primarily in catalysing histone exchange, capable of insertion/removal of dimers containing the histone variant H2A.Z (Awad, Ryan et al. 2010). Deletion has been attributed to redistribution of the H2A.Z histone variant genome-wide (Durand-Dubief, Will et al. 2012). Fun30 has been implicated in maintaining nucleosome positioning proximal to centromeres (Durand-Dubief, Will et al. 2012) and in subsets of genes (Byeon, Wang et al. 2013). Interestingly Fun30 genetically interacts with members of the ORC complex and a double deletion strain of *FUN30* and *orc5-1* exhibits an alteration in cell cycle profile whereby cells accumulate in G₁/early S phase with under-replicated DNA at elevated temperatures (Neves-Costa, Will et al. 2009). Additionally Fun30 appears prerequisite for heterochromatin mediated gene silencing *in vitro* (Yu, Zhang et al. 2011) and *in vivo* (Neves-Costa, Will et al. 2009). The human homolog SMARCAD1 has a similar role whereby it associates with PCNA on replication forks facilitating establishment of heterochromatin (Rowbotham, Barki et al. 2011).

Histone chaperones function in gathering and assembling histones into nucleosomes (Burgess and Zhang 2013). Anti-silencing factor 1 (Asf1) and chromatin assembly factor complex (CAF-1) are of particular interest given their S-phase coupled functions. Both are involved in replication coupled chromatin assembly. Asf1 chaperones H3-H4 dimers (Tyler, Adams et al. 1999) and CAF-1 deposits (H3-H4)₂ tetramers onto nascent DNA while associated with the replisome (Stillman 1986, Shibahara and Stillman 1999). Asf1 is thought to pass histones to CAF-1 and has roles in processing (H3-H4)₂ tetramers during replication through its ability to split the tetramer (English, Adkins et al. 2006), allowing regulation of nucleosome composition on nascent DNA.

Deletion of either chaperone leads to changes in gene expression (Zabaronick and Tyler 2005). *In vitro*, Asf1 has been shown to influence promoter nucleosome positioning at various characterised promoters such as *HO*, *PHO5* and *PHO8* (Korber, Barbaric et al. 2006, Gkikopoulos, Havas et al. 2009). This has been attributed to the role of Asf1 in promoting Rtt109 dependent acetylation of H3K56 (Driscoll, Hudson et al. 2007), a modification with replication specific and independent roles *in vivo* (Schneider, Bajwa et al. 2006, Adkins, Carson et al. 2007). H3K56 acetylation is a histone modification that peaks during S-phase and is thought to facilitate nucleosome assembly (Masumoto, Hawke et al. 2005). Asf1 also associates with RNAPII (Schneider, Bajwa et al. 2006) and facilitates acetylation of H3K56 at promoters (Rufiange, Jacques et al. 2007, Kaplan, Liu et al. 2008).

Effects attributed to CAF-1 are observed post replication and suggest it acts specifically during chromatin assembly (Zabaronick and Tyler 2005). Deletion of CAF-1 subunit Cac2 leads to depletion of histones by ~20% coupled with movement of genic nucleosomes distal to the promoter (van Bakel, Tsui et al. 2013). Furthermore

the loss of (H3-H4)₂ tetramer deposition by CAF-1 is coupled with an abrogation in the size and distribution of okazaki fragments (Smith and Whitehouse 2012).

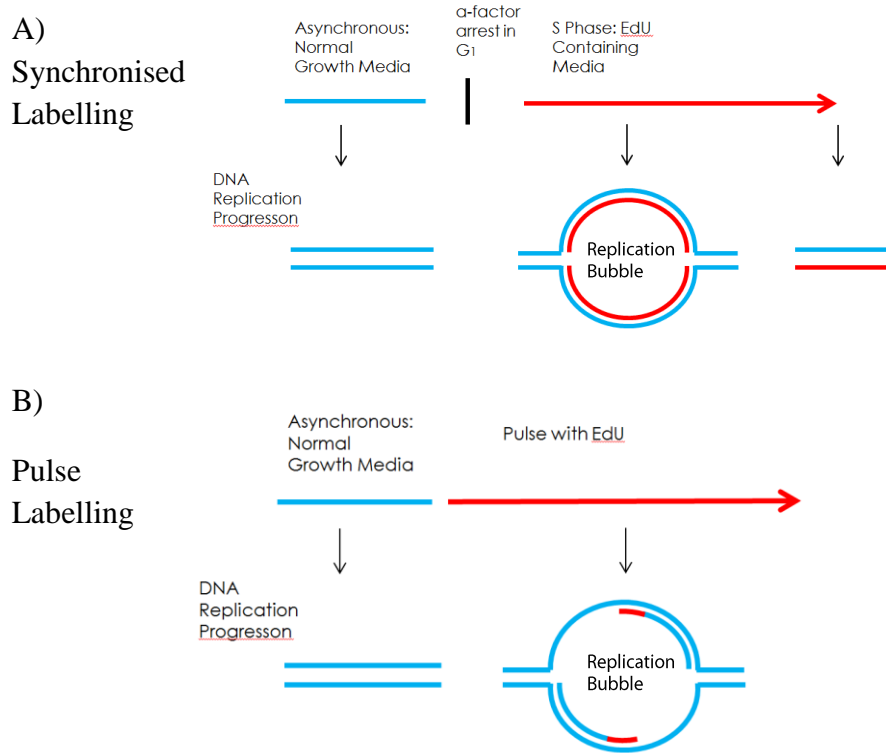


Figure 5-1 – EdU based labelling approaches. **A)** Previously employed approach involves arresting cells in G₁ and subsequent release of cells into replicative medium containing EdU to allow nascent labelling. **B)** Pulse labelling approach involves addition of EdU directly to asynchronous cultures, allowing nascent labelling behind the replication fork in the replicating cell population.

We aimed to study the relative contributions of these factors in shaping nascent chromatin using a pulse labelling approach to isolate a truly nascent nucleosomal DNA. As alluded to in chapter 4, our ability to characterise chromatin maturation appears dependent on isolation of the most immature nascent chromatin possible as nucleosome positioning appears rapidly re-established post replication. To this end we employed a pulse labelling approach by where EdU was added to asynchronous yeast cultures. This allows replicating cells to incorporate the thymidine analogue into nascent DNA directly behind the replisome of elongating replication forks. As a result the age of nascent chromatin is dictated by the length of the EdU pulse chosen.

5.2 Results

5.2.1 Pulse labelled DNA has enrichment to origins of replication

A potential drawback of using EdU pulse labelling in comparison to labelling in synchronised cultures is that there is no way of identifying whether isolated nucleosomal DNA is nascent based on its proximity to known origins. If it is assumed that the rate at which DNA polymerase elongates is constant, one would expect labelling to occur evenly throughout the genome in asynchronous cultures.

When data were analysed following a 10 min pulse, unexpectedly we observe enrichment of pulse labelled nucleosomal reads for the same regions which are enriched when employing EdU in synchronised cultures (figure 5.2). Previously it was demonstrated these regions corresponded to known origins of replication (figure 3.9). In addition nascent pulse labelled reads correlate highly to replication profiles generated from sort-seq data (Pearson correlation 0.862) (Muller, Hawkins et al. 2014).

An interesting, although speculative inference from the data is that replication forks travel faster post firing and their velocity reduces as they migrate distally from replication origins.

Intriguingly, the maxima in the nucleosomal read profile from the synchronised approach appear to slightly anti-correlate with those from the pulse labelling strategy. This hints that the nascent material captured is indeed from elongating replication forks. Given a short, defined labelling timeframe (10min) where the S-phase

population of cells is randomly distributed, it is likely that more EdU labelling will occur at elongating forks rather than at origins that fire during this window.

This differs from nascent labelling in synchronised cultures which captures replicated DNA emanating from firing origins first and results in higher read depth in these regions at early stages of S-phase. If this were the case one would expect the maxima of enrichment from both approaches to overlap.

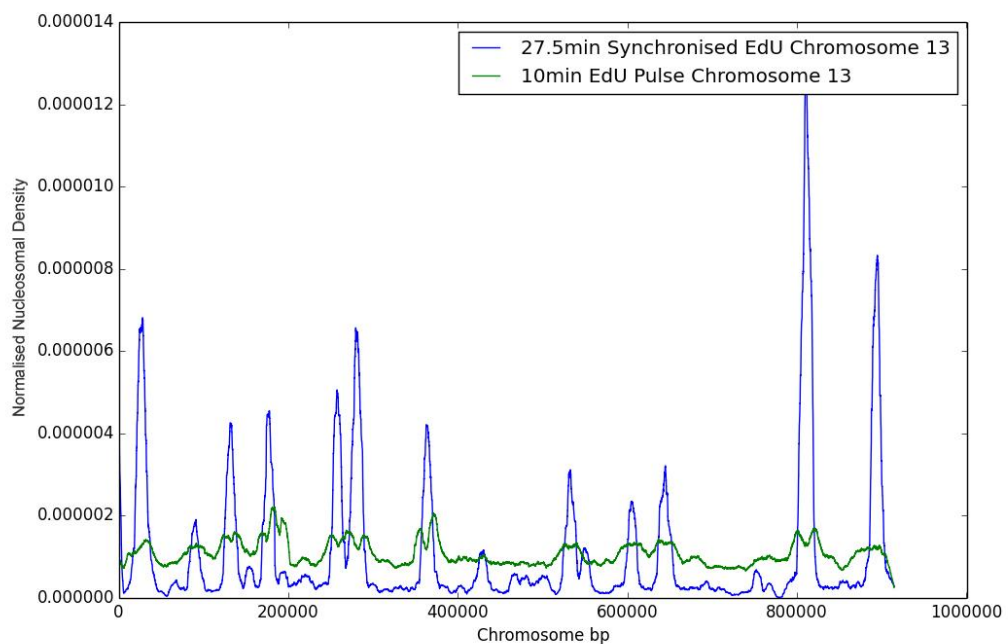


Figure 5-2 – Partial enrichment of pulse labelled nucleosomal DNA to origins of replication. Normalised frequency of nucleosomal dyads per bp along chromosome 13 for a 10min pulse labelled time point (green) shows enrichment of reads along the chromosome which partially correlate with the distribution of nascent reads from an early S-phase time point in a synchronised culture (blue).

5.2.2 Application of the pulse labelling approach to study nascent chromatin structure organised with respect to transcription start sites

The ability to capture nascent chromatin behind the replication fork makes it attractive to re-assess nucleosome organisation along coding regions. This was approached by evaluating pulse labelled nascent nucleosomal DNA from 5, 10 and 50 min time points (figure 5.3).

TSS aligned nucleosome density plots for all genes show nascent chromatin is disrupted in comparison to asynchronous chromatin for the earliest pulse time point (5min, figure 5.3A). A reduction in genic nucleosome density is illustrated by weakened maxima for genic nucleosome oscillations, particularly the +1 nucleosome. Increased nucleosome density in the linker region decreases the ratio between the nucleosomal oscillation maxima and minima, suggesting a more disordered arrangement of nucleosomes along the coding region. Following longer pulses (figure 5.3B-C) mature chromatin states were recovered consisting of progressively better phased arrays of nucleosomes. Evaluation of the nucleosome density profiles on nascent chromatin suggests that the greatest maturation occurs for the +1 nucleosome (figure 5.3D). These observations are consistent with those made using synchronised cultures, but the effects are somewhat more pronounced (figure 4.1 and 4.2).

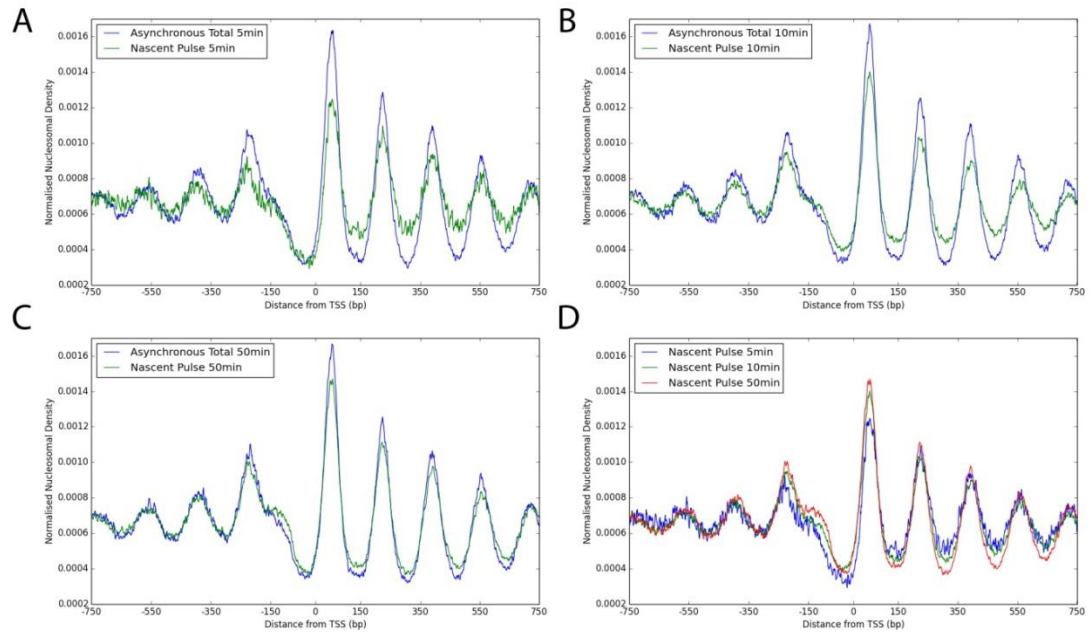


Figure 5-3 - Nucleosome positioning in pulse labelled nascent chromatin aligned to transcription starts sites. The normalised frequency of nucleosome dyads aligned to the TSS of all genes **A)** 5min , **B)** 10 min and **C)** 50 min following addition of EdU to an asynchronously growing culture. Replicated (nascent pulse) and non-replicating (asynchronous total) DNA is shown for each time point. **D)** The normalised frequency of nascent nucleosomal dyads aligned to the TSS of all genes 5, 10 and 50 min post addition of EdU to an asynchronous culture.

5.2.3 Hierarchical clustering reveals pronounced chromatin maturation

Analysis of pulse labelled nascent chromatin structure in coding regions highlighted that direct labelling behind the replisome allows for isolation of a more immature nascent nucleosome organisation when considering all genes (figure 5.3). Despite this, phased genic nucleosomal arrays are apparent, even if slightly disorganised. This suggests that the bulk of chromatin reorganisation following replication has occurred within at least 5 minutes. These results are similar to observations from synchronised cultures (figures 4.1 and 4.2). We endeavoured to identify genes which underwent a more descriptive maturation process using hierarchical clustering (de Hoon, Imoto et al. 2004).

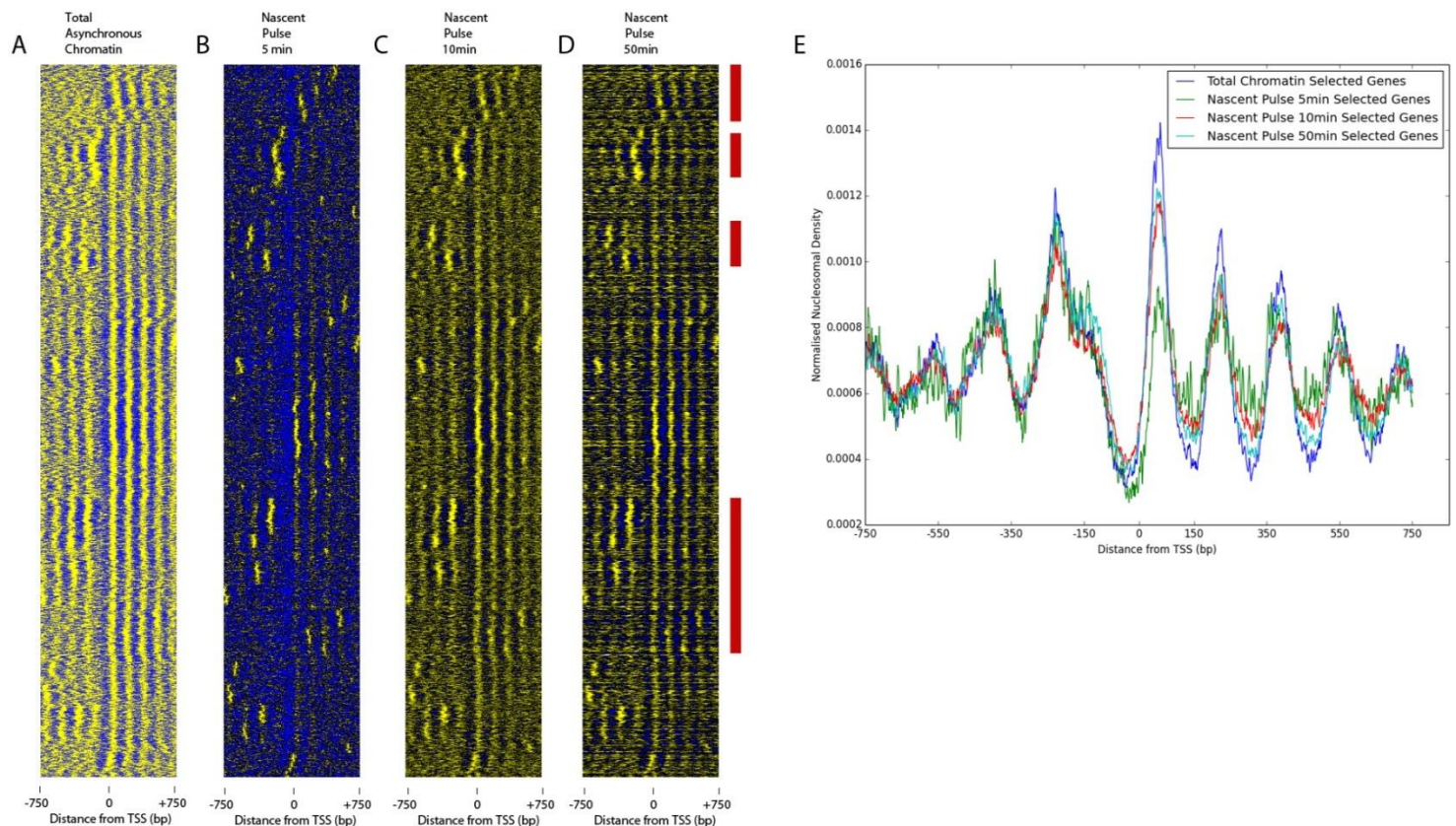


Figure 5-4 - Hierarchical clustering of pulse labelled nascent chromatin. A-D) Heat maps of nucleosomal dyad frequencies aligned to the TSS of all genes. Asynchronous total chromatin along with nascent chromatin pulse labelled with EdU for 5, 10 and 50min time points are shown. Genes are arranged along the y-axis based on the ordering obtained from centroid clustering of the 5min nascent pulse data (B). **E)** Normalised nucleosome density plot aligned to the TSS for selected gene clusters (marked red in D) for non-replicating (total) and replicated (nascent) DNA at specified time points.

The gene organisation obtained by centroid clustering of the 5 min pulse labelled chromatin was applied to subsequent pulse time points in addition to the total chromatin from the asynchronous culture (figure 5.4A-D). Comparison of these heat maps allowed identification of ~2,200 genes (highlighted in red, figure 5.4D) which showed notably abrogated nucleosome organisation at the earliest pulse labelling time point.

The nucleosome density profile for these genes was plotted (figure 5.4E) where the earliest pulse labelled chromatin (5min) shows pronounced disruption in genic nucleosome organisation. This is demonstrated by the severely dampened nucleosomal oscillations of which the +1 nucleosome is most severely affected.

Notably, the maxima of the +1 nucleosome oscillation in the earliest time point appears shifted slightly downstream of the TSS in comparison to the corresponding maxima in the asynchronous total chromatin. This suggests these nucleosomes are initially out of phase post assembly. Strikingly between the 5 and 10 min pulse time points there is a drastic reorganisation of chromatin. Increases in promoter proximal nucleosome occupancy and intensified nucleosomal oscillations are indicative of a maturation towards the arrangement observed for total chromatin in asynchronous cultures. Notably, the increase in +1 nucleosome occupancy is also coupled with repositioning of the +1 nucleosome upstream. That these nucleosome density plots represent almost half of all genes, it would appear that this reorganisation is an accurate portrayal of chromatin organisation post assembly.

Despite employing an approach that allows nascent labelling directly behind the replisome coupled with data analysis that allows extraction of the most severely disrupted chromatin, it appears that nucleosome positioning, to some degree is always maintained along the gene body.

This is likely due to the timeframe in which nascent chromatin can be isolated. While progressively more nascent chromatin isolated demonstrates reduced nucleosome organisation, our approach is limited by the amount of nascent material attainable after such short EdU pulses. Over the course of a 5 min pulse the forces which act on nascent chromatin appear to have arranged phased nucleosomal arrays. The dynamic nature of the maturation process is exemplified when considering the rapid reorganisation over a 5 minute window between pulse time points for selected genes clusters (figure 5.4E). These results are perhaps best explained by a replication coupled chromatin reorganisation that would act to mobilise nucleosomes rapidly on the nascent template long before detection by our methods.

5.2.4 Nucleosome organisation surrounding origins of replication on pulse labelled chromatin

Analysis of nascent chromatin surrounding replication origins isolated from synchronous cultures showed no obvious alterations in chromatin organisation (figures 4.10). A likely explanation is that since DNA replication initiates at these sites, isolated chromatin will have the longest opportunity to mature, resulting in near canonical nucleosome organisation. Hence it was of interest to evaluate whether or not nascent DNA labelling behind the replisome would allow for observation of a more perturbed nucleosome organisation.

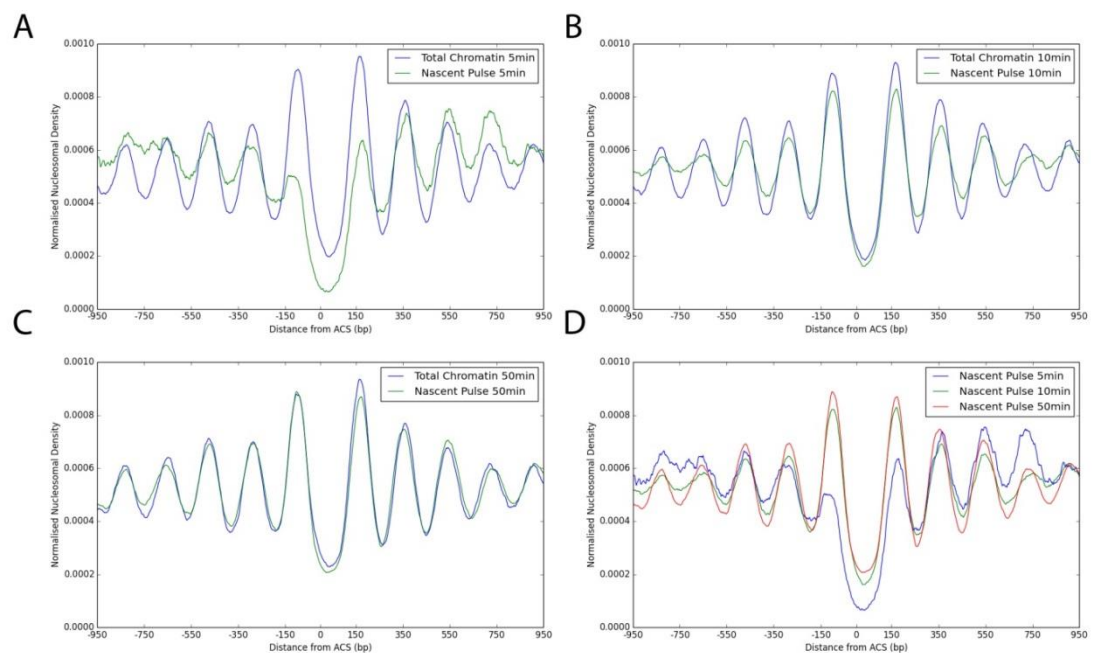


Figure 5-5 - Nucleosome positioning organised with respect to origins of replication on pulse labelled chromatin. The normalised frequency of nucleosome dyads aligned to ACS elements 5min, 10 min and 50 min following addition of EdU to an asynchronously growing culture. **A-C)** Nucleosome density plots for non-replicating (total) and replicated (nascent) DNA at specified time points **D)** Nucleosome density plots for replicated (nascent pulse) DNA at specified EdU pulse time points.

Indeed this appears to be the case when evaluating pulse labelled chromatin aligned with respect to the ACS of replication origins (figure 5.5). In comparison to the asynchronous chromatin, nascent chromatin labelled for 5min has depleted -1 and +1 nucleosome occupancy coupled with irregular nucleosomal oscillations for adjacent nucleosomes (figure 5.5A). Moreover the -1 and +1 nucleosomal oscillation maxima are slightly out of phase with asynchronous chromatin resulting in a larger NDR surrounding the ACS. Maturation in subsequent pulse time points (figure 5.5B) is characterised by dramatic increases in occupancy of the -1 and +1 nucleosomes which are repositioned to canonical locations, leading to a tighter NDR. In addition, nucleosomal oscillation periodicity appears redistributed with respect to the ACS at later time points. Nucleosomes flanking the ACS become strongly positioned and this organisation decays with distance from the ACS bi-directionally (figure 5.5C).

The key event in the maturation process appears to be establishment nucleosomes flanking the ACS which results in canonically phased nucleosomal arrays emanating bi-directionally from the ACS. As highlighted previously, analysis of nucleosome organisation aligned to the TSS has indicated that the typically strongly positioned +1 nucleosome is not retained nor established initially post chromatin assembly. Analysis of chromatin with respect to two genetic boundary elements (TSS and replication origins) hints that strong nucleosome positioning as a result packing against these barriers is late feature of chromatin maturation rather than a pioneering one.

Interestingly at the earliest pulse time point (figure 5.5A) the NDR of nascent chromatin is wider than the total chromatin or indeed nascent chromatin from subsequent time points. This suggests that initially post assembly the ACS flanking nucleosomes are disorganised. Similarly, a wider NDR nucleosome organisation was observed on *in vitro* assembled chromatin at replication origins (Berbenetz, Nislow et

al. 2010). Correct reorganisation may depend upon specific trans acting factors including the ORC complex. Conditional expression of ORC subunits has been shown to be required for correct nucleosome organisation surrounding the ACS, potentially through recruitment of chromatin remodelers or other replication initiation components (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010). Alternatively a rapid replication coupled chromatin reorganisation may establish correct nucleosome spacing that ORC and associated factors are required to maintain.

5.2.5 ATP-Dependent chromatin remodeling ensures canonical nucleosome organisation post replication

Previous results have indicated that nucleosomes are organised on the nascent genic template even after very short periods of DNA replication. This suggests the existence of a replication coupled nucleosome assembly and reorganisation pathway. Mutations to components of this pathway might be expected to specifically affect the replication coupled chromatin ground state rather than the subsequent transcription linked polishing we have previously observed (figures 4.3-4.6). One of the advantages of working in budding yeast is the relative ease with which genetic manipulations can be performed. As a proof of principle we selected three genes that were candidates for involvement in such a replication coupled pathway. We generated yeast strains in which nascent chromatin organisation could be studied in these backgrounds to assess their contribution to what is to date a poorly characterised chromatin organisation pathway.

Fun30 was identified in this regard given it along with human homologue SMARCD1 have been implicated in DNA replication and heterochromatin assembly (Neves-Costa, Will et al. 2009, Rowbotham, Barki et al. 2011). While deletion of the

FUN30 allele does not result in major changes to genome-wide chromatin organisation in asynchronous cultures (Durand-Dubief, Will et al. 2012, Byeon, Wang et al. 2013), this effect may be localised to nascent chromatin. This was evaluated by plotting TSS aligned nucleosome density profiles for pulse labelled nascent chromatin in a *fun30Δ* strain.

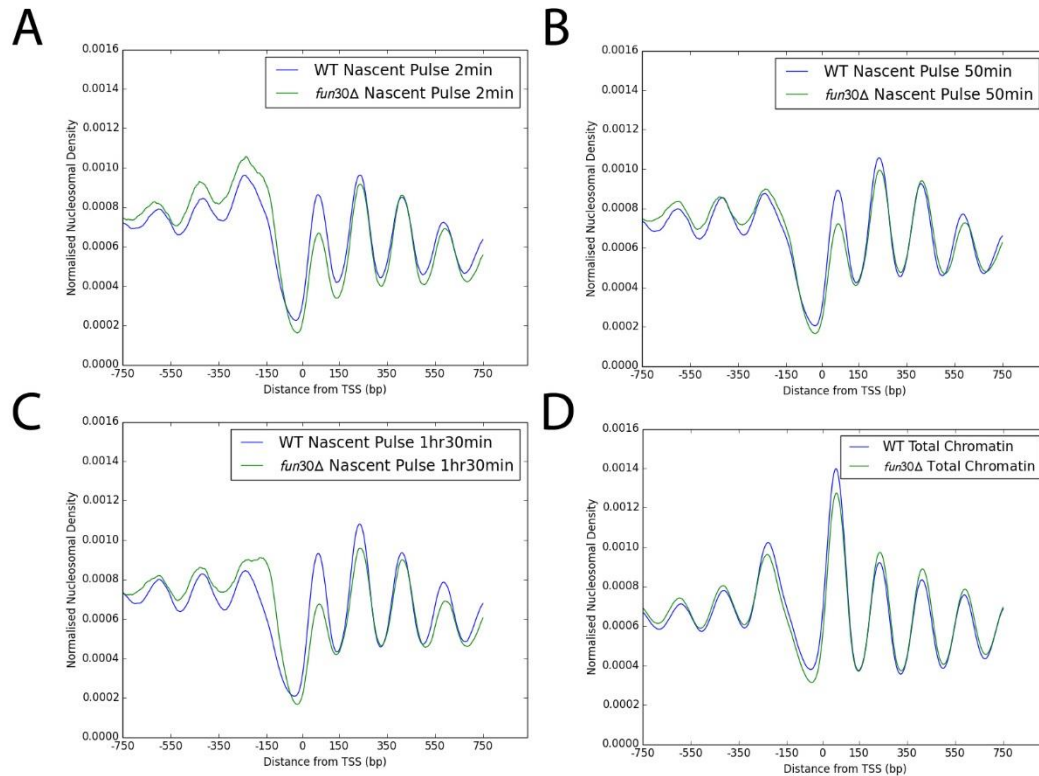


Figure 5-6 - Replication specific roles of Fun30 in nucleosome organisation during DNA replication. Normalised frequency of nascent and non-replicating (total) nucleosome dyads aligned to the TSS of all genes for wild type (WT) and Fun30 deficient strains **A)** 2min , **B)** 50 min and **C)** 1hr 30 min following addition of EdU to an asynchronously growing culture. **D)** The normalised frequency of non-replicating (total) nucleosome dyads aligned to the TSS of all genes for WT and Fun30 depleted strains.

Comparison of wild type (WT) and *fun30Δ* nascent nucleosome density profiles show that at an early pulse time point (figure 5.6A) the +1 nucleosome has reduced occupancy when Fun30 is removed. Unperturbed replication progression is marked by increases in genic nucleosome density which is not observed to the same degree in the absence of Fun30, notably at the +2 and +4 nucleosomes (figure 5.6C). Furthermore

the +4 nucleosomal maxima in the Fun30 mutant is out of phase with that of the wild type indicating a shift in nucleosome positioning on the nascent template (figure 5.6C). These differences are not apparent when comparing chromatin organisation from asynchronous cultures of WT and *fun30Δ* strains (figure 5.6D).

Disruptions to nascent chromatin observed in a *fun30Δ* strain are magnified in the absence of transcription (figure 5.7). Comparison of TSS aligned nucleosome density plots for cell cycle regulated genes between nascent and asynchronous chromatin hints at a Fun30 specific role in nascent nucleosomal array organisation (figure 5.7).

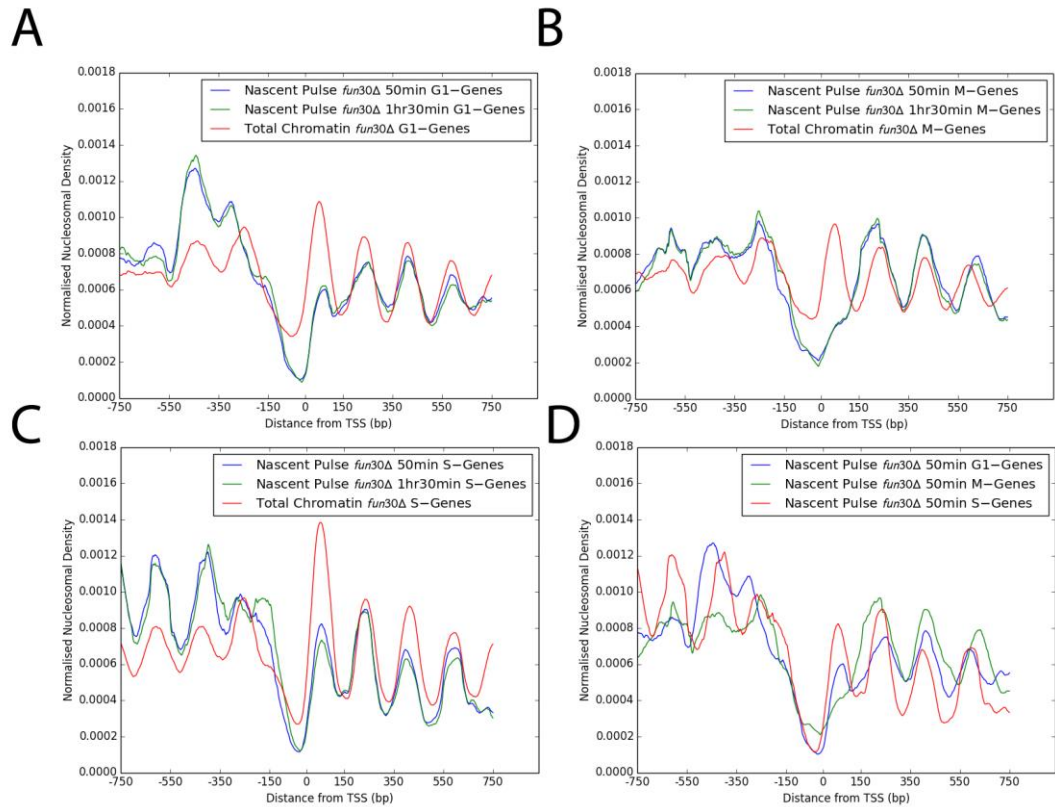


Figure 5-7 - Effect of Fun30 removal on expressed and repressed nascent chromatin. Normalised nucleosomal density plots aligned to the TSS of cell cycle regulated genes which are specifically expressed in **A)** G₁ phase **B)** M phase and **C)** S-phase for replicated (nascent pulse) and non-replicating (total chromatin) DNA at specific EdU pulse time points in a *fun30Δ* mutant. **D)** Comparison of cell cycle specific genes at 50min EdU pulse time point.

G₁ and M phase specific genes (figure 5.7A-B) appear to have a disrupted organisation on nascent chromatin which is not seen on the transcribing S-phase genes (figure 5.7C). G₁ specific genes have notably reduced +1 nucleosome occupancy. Strikingly the +1 appears lost on M phase genes coupled with loss of positioning of +2 and +4 nucleosomes. In contrast, actively transcribing genes retain canonically positioned genic nucleosomal arrays (figure 5.7C) which have acute nucleosomal oscillation maxima/minima (figure 5.7D). These results suggest that separate transcription and replication directed pathways can organise chromatin on the nascent template. Trans-acting factors, such as Fun30 may facilitate the basis of correct nucleosome array formation in a replication coupled manner (figure 5.7). However in the absence of both pathways, nascently chromatin remains disrupted.

5.2.6 Histone supply by histone chaperones is vital for nucleosome organisation on nascent DNA

While histone chaperones do not act directly to position nucleosomes, trafficking and modification of soluble histones facilitates multiple replication dependent and independent processes which have implications for nucleosome positioning (Alabert and Groth 2012, Das and Tyler 2013). Deletion of individual CAF-1 subunits are coupled with histone level depletion (van Bakel, Tsui et al. 2013) while Asf1 is required for maintenance of histone occupancy along highly transcribed genes (Kim, Seol et al. 2007). Given that our pulse labelling strategy allows labelling behind the replication fork where these chaperones act, their contributions towards nascent chromatin assembly and maturation may be more finely examined.

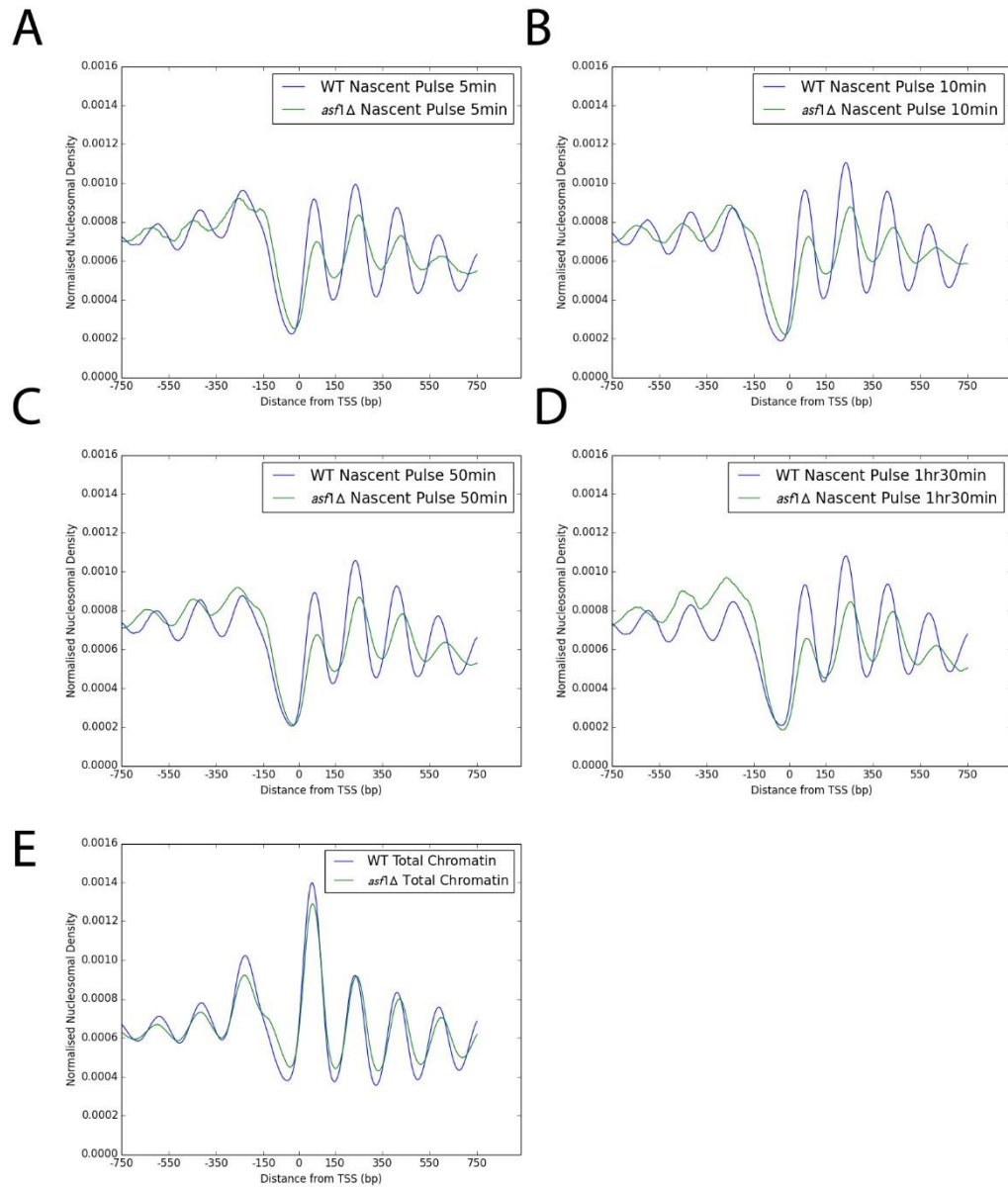


Figure 5-8 - Replication specific roles of Asf1 in nucleosome organisation during DNA replication. Normalised frequency of nascent nucleosomal dyads aligned to the TSS of all genes for wild type (WT) and Asf1 deficient strains **A)** 5min , **B)** 10 min , **C)** 50 min and **D)** 1hr 30 min following addition of EdU to an asynchronously growing culture. **E)** The normalised frequency of non-replicating (total chromatin) nucleosomal dyads aligned to TSS of all genes for wild type (WT) and Asf1 depleted strains.

Indeed, comparison of wild type (WT) and *asf1Δ* nascent nucleosome profiles (figure 5.8) show that organisation is severely impaired on nascent chromatin in the chaperone mutant. A notable reduction in all genic nucleosomes density is apparent coupled with shifts in +2 and +3 nucleosomal maxima, indicative of a shift in positioning.

Positioning of the +4 nucleosome is almost completely ablated as illustrated by the loss of a prominent nucleosomal oscillation (figure 5.8).

In contrast, nucleosome profiles from asynchronously growing *asf1* Δ strains appear canonically phased with no great loss of nucleosomal occupancy. This agrees with previous observations of chromatin organisation in *asf1* Δ depleted strains (van Bakel, Tsui et al. 2013). That nucleosome density profiles for *asf1* Δ are only disrupted on nascent chromatin suggests that Asf1, either directly through facilitating replication coupled H3K56 acetylation or indirectly through maintenance of histone levels is vital for initial nucleosome array organisation on replicated DNA.

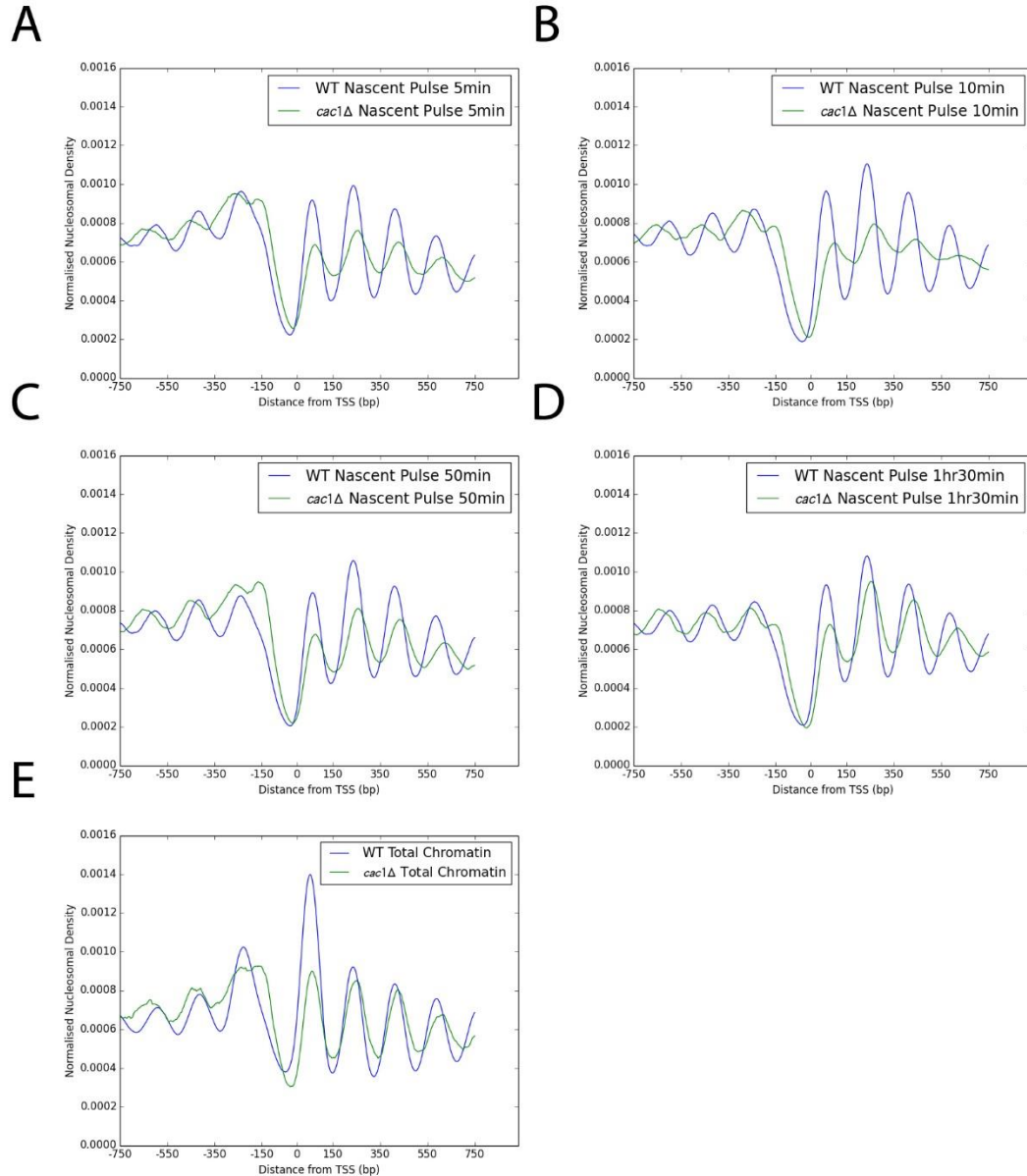


Figure 5-9 - Replication specific roles of Cac1 in nucleosome organisation during DNA replication. Normalised frequency of nascent nucleosomal dyads aligned to the TSS of all genes for wild type (WT) and Cac1 deficient strains **A)** 5min , **B)** 10 min **C)** 50 min and **D)** 1hr 30 min following addition of EdU to an asynchronously growing culture. **E)** The normalised frequency of non-replicating (total chromatin) nucleosome dyads aligned to the TSS of all genes for WT and Cac1 depleted strains.

Deletion of CAF-1 subunit Cac2 has been characterised by shifting of genic nucleosomes distal to the TSS, becoming more pronounced for downstream nucleosomes (van Bakel, Tsui et al. 2013). A similar scenario is seen for *cac1Δ* depleted strains grown in asynchronous culture (figure 5.9F).

These differences are magnified on nascent chromatin. Pulse labelled chromatin (figure 5.9A-B) displays pronounced shifts in all coding region nucleosomes distal to the TSS with a near ablation in +4 nucleosome positioning at earlier time points. Replication progression (figure 5.9C-D) is marked by increased genic nucleosome occupancy and increased amplitude in nucleosomal oscillations, indicative of improved nucleosome organisation despite the fact that they remain out of phase with wild type locations.

Nucleosome density profiles for CAF-1 and Asf1 chaperone mutants on nascent chromatin have largely overlapping characteristics. Both illustrate reduced genic nucleosome occupancy coupled with promoter distal shifts in genic nucleosome positions. While Asf1 has various functions it is likely that the observed effects are due to histone level depletion as these are the common functions of both chaperones (Kim, Seol et al. 2007, van Bakel, Tsui et al. 2013). Similarly cells lacking Asf1 display global changes in transcription similar to those seen in cells lacking Cac2 or histone H4 (Zabaronick and Tyler 2005). However, such inferences are speculative since histone levels on nascent chromatin in these experiments are not known for either chaperone.

5.2.7 Histone supply is crucial for nucleosome organisation in the absence of transcription

In the histone chaperones tested, defects to TSS aligned nucleosome organisation appear greatest on nascent chromatin. This suggests that the activities of these chaperones can be compensated for later on in the chromatin maturation programme, potentially in a transcription directed manner. Previously we identified a subtle role for transcription in chromatin maturation in synchronised cultures (figure 4.3-4.6) but a more important role in the absence of Fun30 (figure 5.7). This prompted us to

consider nascent nucleosome organisation in genes which are transcriptionally active and silent. This was achieved using genes whose expression is specific to stages of the cell cycle (Rowicka, Kudlicki et al. 2007).

Comparisons of TSS aligned nucleosome density plots for cell cycle specific genes between nascent and asynchronous chromatin were made for *asf1Δ* and *cac1Δ* strains (figure 5.10 and 5.11).

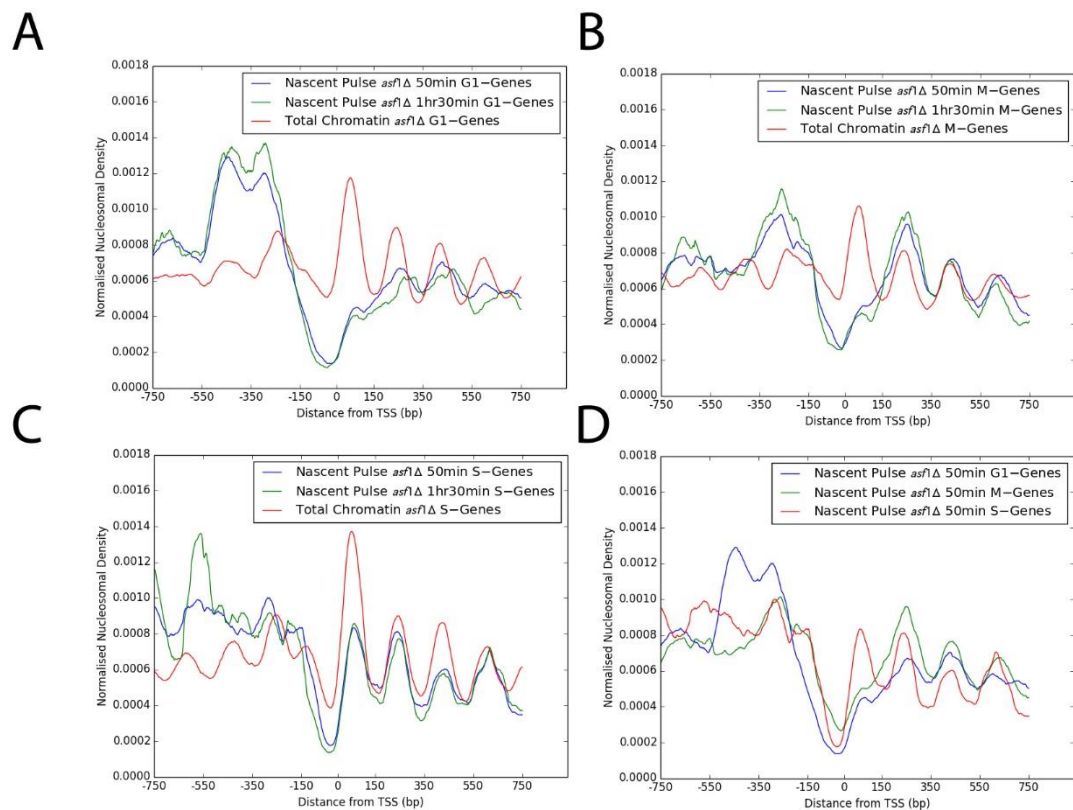


Figure 5-10 - Effect of Asf1 removal on expressed and repressed nascent chromatin. Normalised nucleosome density plots aligned to the TSS of cell cycle regulated genes which are specifically expressed in **A)** G₁ phase **B)** M phase and **C)** S-phase for replicated (nascent pulse) and non-replicating (total chromatin) DNA at specific EdU pulse time points in an *asf1Δ* mutant. **D)** Comparison of cell cycle specific genes at 50min EdU pulse time point.

Strikingly, there are drastic alterations to nascent genic nucleosome organisation in an *asf1Δ* mutant for non-transcribing genes (G₁ and M phase) in comparison to total chromatin from asynchronous cell populations (figure 5.10A-B). The +1 nucleosome

occupancy and positioning is completely ablated coupled with shifts in downstream nucleosome positioning in G₁ specific genes. Moreover, organisation upstream of the promoter appears disrupted with a distinct increase in occupancy between the -1 and -2 nucleosomes. However these effects are not apparent for transcribing S-phase specific genes (figure 5.10C). While having reduced occupancy, all genic nucleosomes are present along the gene body and appear relatively well phased in comparison to the chromatin from asynchronous cultures.

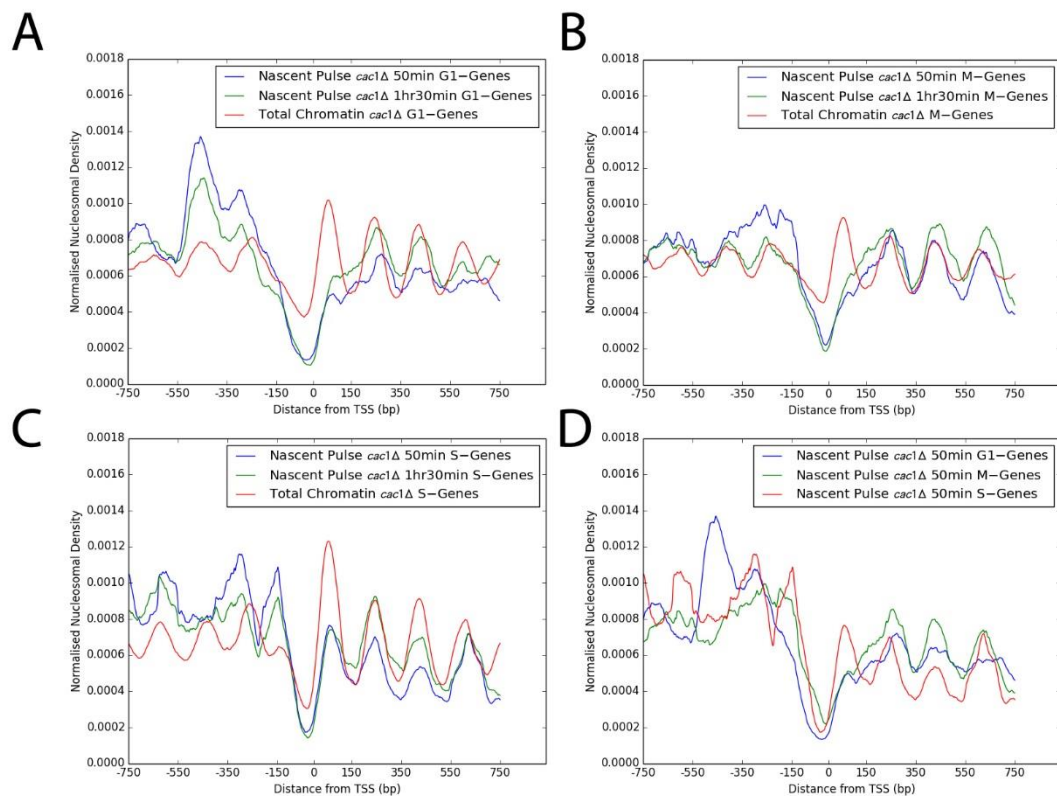


Figure 5-11 - Effect of Cac1 removal on expressed and repressed nascent chromatin. Normalised nucleosome density plots aligned to the TSS of cell cycle regulated genes which are specifically expressed in **A)** G₁ phase **B)** M phase and **C)** S-phase for replicated (nascent) and non-replicating (total chromatin) DNA at specific EdU pulse time points in a *cac1Δ* mutant. **D)** Comparison of cell cycle specific genes at 50min EdU pulse time point.

Similar observations can be made when considering a *cac1Δ* mutant (figure 5.11).

Again loss of the +1 nucleosome coupled with shifts in positioning are observed on

nascent chromatin for non-transcribing genes (figure 5.11 A-B) while transcribing genes (figure 5.11C) contain all genic nucleosomes with pronounced nucleosomal oscillations (figure 5.11D).

Results from both chaperones support findings observed in a strain depleted of Fun30 in suggesting that at least two mechanisms can organise nucleosomes on nascent DNA. The replication coupled pathway may fail in the absence of adequate supply of histones by chaperones. Depleted histone levels may lead to reduced numbers of nucleosomes which form a more diffuse arrangement along the nascent template. This organisation could latterly be redistributed to a typical genic nucleosome arrangement by trans-acting factors such as remodeling enzymes that are coupled to a subsequent transcription event.

5.3 Discussion

Characterisation of nascent chromatin isolated from synchronous cultures hints that genic nucleosome organisation becomes less ordered on the most nascently replicated DNA (figure 4.8). This led us to consider a scenario where nascent chromatin is significantly disorganised behind the replisome prior to rapid reorganisation. In order to view nascent chromatin during this window we employed a pulse labelling strategy by where exogenous EdU added to asynchronous cultures allows nascent labelling in replicating cells directly behind randomly distributed replication forks (figure 5.1). In principle pulse labelled chromatin can only be as old as the length of the pulse chosen.

An anticipated frailty in this approach was its validation in comparison to synchronised methods. Considering an asynchronous culture in which replicating cells are at various stages of S-phase one would presume replication forks to be randomly distributed across the genome. Hence pulse labelling should result in equal distribution

of nascent reads across the genome and would prevent comparison of nascent read distributions to previously annotated DNA replication profiles (Yabuki, Terashima et al. 2002, Muller, Hawkins et al. 2014). Surprisingly, pulse labelled nucleosomal DNA appears partially enriched at replication origins. This profile of nascent read enrichment is not dissimilar to that obtained from EdU labelling in synchronised cultures (figure 5.2). This observed enrichment correlates well with replication profiles generated from the S-phase population of asynchronous cells sorted by FACS (Muller, Hawkins et al. 2014). Suggested speculatively, these findings would suggest that over the course of a 10 min pulse (figure 5.2) that replication forks travel faster proximal to origins allowing greater EdU enrichment behind the replisome in comparison to regions distal to origins. Differences in fork velocity have been reported previously (Raghuraman, Winzeler et al. 2001), however DNA combing in mammals has suggested forks emanating from the same origins have constant velocity (Conti, Sacca et al. 2007). Variation in fork velocity appears related to origin density and is inversely related to the number of active replication forks, which is determined by the level of origin firing (Spiesser, Diener et al. 2010, Zhong, Nellimoottil et al. 2013). In contrast, our data suggests replication forks have velocities that decay with distance from origins. However, this interpretation is conflict with ChIP studies of replisome components (Azvolinsky, Giresi et al. 2009, Sekedat, Fenyo et al. 2010). These studies imply that replication forks maintain a constant velocity (de Moura, Retkute et al. 2010, Yang, Rhind et al. 2010). While it is not the main intention of this study, the interpretations made regarding fork rates are made using rather blunt analysis and it would be of considerable interest to fine tune our observations in the future.

Assessment of pulse labelled nascent chromatin largely reflects observations made using a synchronised approach (figure 5.3). Nascent nucleosome density profiles again are characterised by attenuated nucleosomal oscillations which recover amplitude during the course of replication, indicative of improved spacing on the nascent template. Concurring results by three different approaches gives us confidence that the nascent nucleosome profiles observed describe the general process of chromatin maturation. However, that a basic genic nucleosome organisation appears already established after 5 min suggests chromatin maturation is so rapid that only isolating the most nascent nucleosomal fragments would provide a representative view of truly nascent chromatin. Given the trends observed it is likely that progressively earlier time points will yield more disorganised nucleosome organisation. Unfortunately such experiments are hampered by an inability to reliably isolate nascent nucleosomal DNA from earlier pulse labelling time points.

Our views of nascent chromatin maturation provide insights towards a model of how nucleosomes are ordered genome-wide, of these a barrier packing model is favoured (Zhang, Wippo et al. 2011). Clustering (figure 5.4) identified a large (~ %50) subset of genes which have abrogated nucleosomal arrangements at the earliest pulse time point. Notably, at the most nascent time point for these genes, the +1 nucleosome read depth is severely reduced and its nucleosomal maxima appears out of phase suggesting a shift in positioning. These observations are consistent with clustering performed on nascent chromatin during a synchronised S-phase (figure 4.7). A model where nucleosomes actively pack against a barrier at the NDR would implicate that the +1 nucleosome occupancy be retained or established initially on the nascent genetic template as observed during chromatin reconstitution experiments (Zhang, Wippo et al. 2011). These data provide further evidence that downstream genic nucleosome

organisation appears to occur independently of +1 nucleosome organisation. These results support the observation made by the Nislow group that genic nucleosomes can in fact be organised independently (van Bakel, Tsui et al. 2013).

In addition to the TSS, specific nucleosome organisation occurs at origins of replication. These contain promoter like, nucleosome excluding DNA sequences (Berbenetz, Nislow et al. 2010, Eaton, Galani et al. 2010) which are flanked by two well positioned nucleosomes that appear to facilitate symmetrical arrays of nucleosomes in 5' and 3' directions. Surprisingly, -1 and +1 flanking nucleosomes regain their occupancy as a late event of chromatin maturation (figure 5.5). Similar to genic nucleosome organisation, one would anticipate that these nucleosomes would be retained or at least recuperate occupancy first if nucleosomes were actively packed against this genomic barrier. These findings would further argue against barrier packing as a model to describe nucleosome organisation with respect to genetic elements.

Our studies of nascent chromatin highlighted that nucleosome reorganisation is a rapid process hinting that this process is replication coupled. We aimed to dissect elements of this uncharacterised pathway by extending our study of nascent chromatin to the ATP-dependent chromatin remodeler Fun30 and histone chaperones Asf1 and CAF-1. Given their roles in DNA replication, they may have roles specific to nascent chromatin organisation.

Indeed this appears to be the case for Fun30. Comparison of TSS aligned nascent nucleosome density profiles between wild type and the deletion strain indicates notable loss in organisation of the +1 and +4 genic nucleosomes which is not observed to the same extent in asynchronous cultures (figure 5.6).

Even more dramatic aberrations in nucleosome organisation are observed on nascent chromatin from Asf1 and CAF-1 mutant strains (figures 5.8, 5.9). Downstream genic nucleosomes have reduced read depth and appear out of phase with wild type nascent chromatin. It would appear unlikely that the role of Asf1 in promoting Rtt109 mediated H3K56 acetylation (Schneider, Bajwa et al. 2006) is having a direct influence on the observed nascent nucleosome organisation. Given the shared functions of Asf1 and CAF-1 it is likely that the similar nucleosomal density profiles observed are due to loss of histone supply to the nascent genetic template given both chaperones have been implicated in governing histone levels (Kim, Seol et al. 2007, van Bakel, Tsui et al. 2013).

Analysis of chromatin organisation in these mutants strongly suggests their roles in nucleosome organisation are S-phase coupled. Alterations to nascent nucleosome organisation in these mutants are not observed to the same degree in asynchronously growing strains. This suggests that their roles in a replication coupled nucleosome assembly and spacing mechanism are compensated latterly. Our previous results indicated that nucleosome organisation was largely established independently of transcription (figures 4.3-4.6). However, there remained the possibility that aberrantly assembled chromatin could be rescued by a transcription directed pathway.

Remarkably, absence of transcription coupled with loss of chaperone or Fun30 activity results in dramatic alterations to chromatin structure. This is indicated by loss of specific genic nucleosomes and in certain instances a complete loss in organisation altogether (figure 5.10A,5.11A). Reduction in histone content associated with loss of chaperones may result in a more openly spaced, random nucleosome organisation on the non-transcribed nascent template. Importantly, transcribing genes have all phased genic nucleosomes (albeit with reduced occupancy). This would indicate that the

activity of a transcription coupled pathway could take over the role of nucleosome organisation from the replication coupled mechanism if it fails to establish correct organisation.

6 Conclusions and future outlook

The aim of this project was to map nucleosome positions on nascent chromatin during DNA replication. Studying nascent nucleosome distributions presents an opportunity to study the relative contributions of factors known to influence nucleosome architecture during chromatin maturation in addition to evaluating models that explain genic nucleosome positioning.

Nucleosome positioning was studied in nascent chromatin from synchronised cultures isolated by CsCl gradient ultracentrifugation and incorporation of the thymidine analogue EdU. Nucleosome density profiles aligned with respect to all genes suggests a subtle maturation occurs but that chromatin organisation is largely retained on the nascent template. We found that a pulse labelling approach carried out in asynchronous cells could most effectively enrich for nascent chromatin.

All three approaches provide evidence that chromatin is significantly reassembled in the minutes following replication. Rapid chromatin reorganisation post replication is consistent with classical observations of chromatin reassembly behind the replication fork (McKnight and Miller 1977, Sogo, Stahl et al. 1986) and retention of nucleosome positioning on the nascent template (Lucchini, Wellinger et al. 2001). In fact these studies indicate that reassembly and positioning of nucleosomes occurs within seconds.

Chromatin reorganisation on these time scales is so rapid that few genes can have been transcribed (Pelechano, Chavez et al. 2010). Yet the nucleosomal pattern is organised with respect to transcription start sites. This would be best explained if some factors re-engage with promoter DNA very rapidly following DNA replication and that these

generate a reference point from which nucleosomal arrays are propagated over the coding region along the lines proposed in the barrier packing model (Zhang, Wippo et al. 2011).

If there is a distinct replication coupled chromatin assembly and organisation pathway, then it might be anticipated that interfering with components of this pathway would result in slow chromatin organisation following replication and perhaps greater dependence on a separate transcription coupled assembly pathway. To test this concept further, chromatin replication was studied in strains in which candidate genes *ASF1*, *FUN30* and *CAC1* had been deleted. Loss of any of these factors (notably the histone chaperones) results in greater disorder within newly replicated chromatin. This effect was enhanced at genes transcribed at low levels. This suggests that a failure to reorganise chromatin following DNA replication can be compensated for by transcription directed organisation.

The evidence for the existence of separate replication and transcription coupled packaging raises the possibility that nucleosome reorganisation is carried out for short and precisely regulated periods of time. Restricting the duration during which nucleosome spacing occurs would provide a means of reducing the energy consumed, while ensuring that chromatin was reorganised following disruption immediately following the transit of DNA and RNA polymerases. The precise temporal regulation of nucleosome spacing also has the potential to provide explanations for observed features in nascent chromatin that are otherwise difficult to reconcile with the barrier packaging model.

Previously, Nislow and co-workers (van Bakel, Tsui et al. 2013) observed that subsets of genes could be identified where nucleosomes were missing or misaligned with the

canonical nucleosomal pattern over coding regions. In these cases, there was no effect of miss positioned or missing nucleosomes on the locations of adjacent nucleosomes. In this study we have made similar observations. Hierarchical clustering revealed cohorts of genes that had reduced genic nucleosome density at the +1 location, yet retained organisation at locations further 3'. In addition following mutation to genes involved in replication coupled assembly the +1 nucleosome was severely depleted at early time points at genes not expressed in S-phase. However, some organisation was retained at positions further 3'. All of these observations are difficult to reconcile with the barrier packing model which predicts that the positioning of a particular nucleosome is dependent on the location of its 5' neighbour.

If the barrier packing model is combined with tight temporal regulation the situation changes. For example, if a nucleosome is lost following a short burst of repositioning either coupled to DNA replication or transcription, this would not have an impact on the positioning of surrounding nucleosomes. Furthermore, if there is a loss of organisation following replication, this could potentially be restored during subsequent transcription coupled reassembly. That nucleosome spacing enzymes can rapidly complete chromatin assembly and spacing is supported by findings from the Kadonaga lab that showed organisation of nucleosomes on one template is completed before organisation on another is started (Torigoe, Urwin et al. 2011).

If replicated DNA is assembled with a lower density of histones the barrier packing model does not anticipate that nucleosome spacing will increase (Zhang, Wippo et al. 2011). However, analysis of chromatin organisation during H4 depletion indicates that this is not entirely accurate (van Bakel, Tsui et al. 2013). This has been taken as evidence that there needs to be a modification to the barrier packaging model. Previous studies indicate that nucleosome density is reduced by ~20% in a CAF-1 mutant (van

Bakel, Tsui et al. 2013). Consistent with observations made by Nislow and colleagues, we observed that spacing on nascent chromatin is increased to ~174bp in *asf1Δ* and *cac1Δ* mutant backgrounds. Notably, we observe drastic alterations to nascent nucleosome spacing on transcriptionally silent genes in CAF-1 and Asf1 mutants. As this reduction in nucleosome density is not so evident within mature chromatin, it is possible that a distinct transcription coupled chromatin packaging pathway acts to correct the spacing defect. Outside of S-phase the transcription coupled pathway may effectively scavenge the limited supply of histones and assemble tracts of nucleosomes with normal density and spacing over coding regions. After these have been assembled, histone turnover could result in the loss of nucleosomes from some locations, but with limited supply reassembly might be less efficient. This scenario could give rise to the observed regular spacing but reduced nucleosome occupancy following histone depletion.

Another question that arises from the studies of chromatin in the mutant strains is why the +1 nucleosome appears particularly unstable. In the case of Fun30 this could relate to functions relating to H2A.Z. The distribution of this variant is perturbed in a Fun30 mutant (Durand-Dubief, Will et al. 2012) and H2A.Z is normally found adjacent to promoters, especially within the +1 nucleosome. Such links do not exist for CAF-1 and Asf1. In these strains it may be that a reduced histone supply limits the rate of chromatin reassembly. Under such conditions it might be anticipated that at regions of high nucleosome turnover, occupancy would be reduced due to a lower rate of reassembly. As promoter proximal nucleosomes are sites of high turnover, this may explain why the +1 nucleosome is affected to such an extent (Dion, Kaplan et al. 2007).

If the activity of remodeling enzymes is precisely regulated what factors could act to co-ordinate this regulation? One possibility is that remodeling enzymes would be physically coupled to the relevant polymerase. The strongest evidence for this relates to coupling between RNA polymerase and Chd1 via the PAF complex (Simic, Lindstrom et al. 2003). In the case of DNA polymerase SMARCAD1 has been shown to associate with PCNA during replication to facilitate heterochromatin formation (Rowbotham, Barki et al. 2011).

An alternative means via which such a coupling could be provided would be if the activity of remodeling enzymes was precisely regulated by post translational modifications to histones. In the case of DNA replication, such modifications would be anticipated to generate an “ON” signal in nascent disorganised chromatin. There would be an additional requirement that this “ON” signal was converted to an “OFF” signal once nucleosomes were organised. Again this could potentially occur through the addition or removal of a post-translational modification to histone proteins, but the activity could be sensitive to nucleosome spacing. Interestingly there is a precedent for such an activity (Lee, Wu et al. 2013). In addition the metazoan NuRD complex combines nucleosome remodeling and histone deacetylase activities (Xue, Wong et al. 1998, Zhang, LeRoy et al. 1998) and is likely to act such that it could potentially sense adjacent nucleosomes (Lee, Wu et al. 2013).

The system we have established to monitor nascent chromatin provides a means of assessing the contributions of candidate proteins. Removal of the “OFF” signal would be anticipated to result in hyper organised chromatin and reversal of some of the defects observed on nascent chromatin in the chaperone mutants. In future we hope to apply this to study the contributions of enzymes involved in histone acetylation, methylation and ubiquitination.

7 References

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